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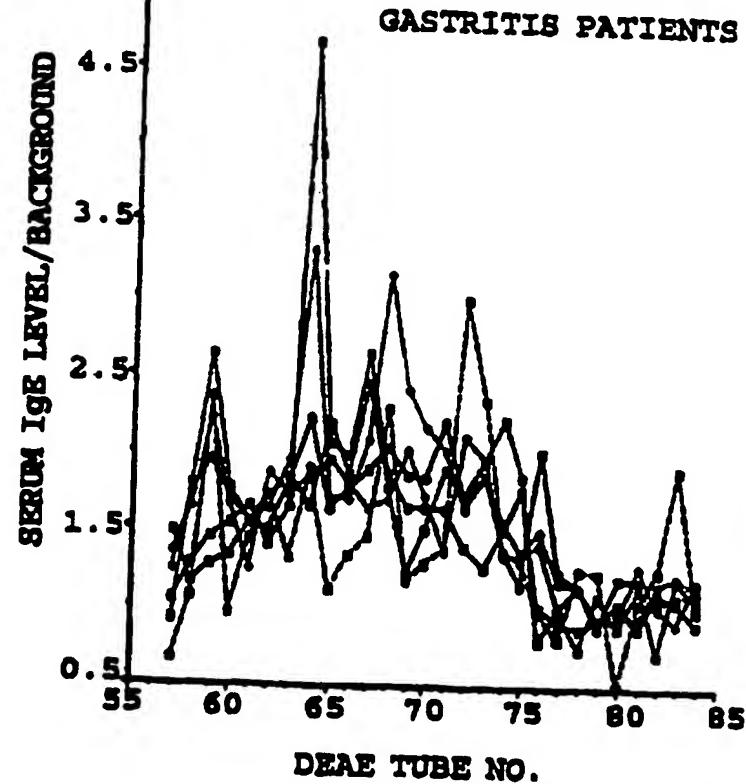
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WITH ANTIGENS OF MICROORGANISMS

(57) Abstract

A library of isolated and purified antigens specific for a micro organism is a set of individual molecules. The library forms antigen-antibody complexes useful in the context of diagnosing, vaccinating, treating conditions or diseases associated with a specific microorganism such as *Helicobacter pylori*-induced gastroduodenal disease. For the antigen-antibody complexes in question the antibody is an immunoglobulin, which is IgE if the antigens are allergens. Complexes with IgA, IgG and IgM also are useful for monitoring progression of the disease and therapy effectiveness and selecting antigens for use in a customized vaccine. By this multivariate approach, a specific condition is diagnosed with high sensitivity and specificity by determining whether complexes form between a specific antigen library and a biological sample which contains immunoglobulins from an individual. In addition, such libraries are useful for immunotherapy, particularly with respect to antigen desensitization and to production of customized vaccines.



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METHODS AND COMPOSITIONS FOR PRODUCTION OF  
CUSTOMIZED VACCINES FOR DISEASES ASSOCIATED  
WITH ANTIGENS OF MICROORGANISMS

BACKGROUND OF THE INVENTION

The present invention relates to methods and compositions for the production of customized vaccines for diseases associated with antigens of microorganisms.

5       A number of idiopathic recurrent diseases are of unknown etiology. Some of these diseases are believed to be linked to infection by a microorganism, for example, a bacterium. Yet the causal relationship between the microorganism and the disease is not established for many  
10      of these diseases or conditions. Even in diseases or conditions showing an association with an infectious agent, the etiology leading to the disease symptoms is generally unknown. For some diseases, such as chronic gastritis and peptic ulcer disease and chronic  
15      inflammatory diseases of the nose and paranasal sinuses, a link is suspected between infection and allergy. The initiating event is suspected to be an infection, with allergy developing as a sequel. Subsequently, infection may exacerbate the microbial allergy which leads to both  
20      chronic hypersensitivity and chronic infection. Data in support of these theories is not capable of discriminating between them.

It has been suggested that bacterial allergy may play a significant role in chronic diseases of the aerodigestive tract. Examples of aerodigestive diseases potentially effected by bacterial hypersensitivity include asthma, nasal polyps, chronic gastritis and gastric ulcer disease. At present no uniform view exists as to how the allergic process is mediated or, more precisely, how mast cell degranulation is induced. A bacteria-specific, IgE-mediated response is postulated for some diseases in this category.

IgE-mediated reactions resulting in chronic inflammation rather than acute, short-lived reactions have been well described. The hallmark event described in these studies is mast cell degranulation. Mast cells 5 release vasoactive mediators and late-phase reactants, such as chemotactic agents, recruit neutrophils, eosinophils, and monocytes. The influx of these cells is followed by lymphocytic infiltration. These events may become part of a chronic, repetitive process through the 10 maintenance of a protracted mast cell degranulation.

Digestive diseases include the related disorders of chronic gastritis and peptic ulcer disease which appear to be associated with the microorganism *Helicobacter pylori*, but the nature of the association, and the 15 mechanisms linking infection with subsequent symptoms are not known (Megraud et al., 1993). Unraveling the etiology of these diseases is important because chronic gastritis and peptic ulcer disease are diseases of major significance. *Helicobacter pylori* may be the most common 20 worldwide infection present in about 50% of adults in developing countries. For example, the prevalence of infection in the 40-49 age group in Japan was 80 to 90%. Infection of children with *H. pylori* persists through their lifetime and predisposes them to stomach and 25 duodenal disease. The risk of gastric cancer attributable to *H. pylori* is estimated at 60% (Parsonnet et al., 1991). In many countries of Asia and Latin America, gastric cancer is the most common malignancy among men and second most common among women. The use of 30 screening and preventive treatment would be enhanced if populations at high risk for cancer could be pinpointed. This is effected by the methods and compositions of the present invention. Five to ten percent of all individuals develop chronic gastritis and/or 35 gastroduodenal ulcers in their lifetime. Ulcer disease is a common cause of morbidity. The annual prevalence of symptomatic peptic ulcer disease in the United States of America is approximately 18 per 1,000 adults (or about

4,500,000 people). Approximately 350,000 new cases of peptic ulcer disease are diagnosed each year in the U.S.

5 Diagnosis of *H. pylori*-associated diseases is usually performed by gastroduodenal endoscopy, an invasive and costly procedure. A breath urea test also is available. However, combined sensitivity-specificity is not 100% (Graham et al., 1988). Other disadvantages are that the test requires expensive equipment and ingestion of radioactive materials, a procedure to be avoided if 10 possible. Treatment encompasses oral medication, dietary controls, and surgery. Rarely is treatment successful in effecting a "cure," rather these chronic conditions are characterized by cycles of improvement and relapse.

15 Since the report by Marshall (1983) that the bacteria *H. pylori* is physically associated with the lesions of chronic gastritis, a great deal of work has been done in an effort to elucidate a causal relationship between the organism and the chronic disease. Early speculations regarding localized pH changes induced by *H. pylori*, the 20 release of toxins (Hupertz et al., 1988) and destructive enzymes (Slomiany et al., 1989), and the differences between different strains of the bacteria (Eaton et al., 1989) have not engendered firm conclusions, accepted in the art, concerning the etiology of the disease 25 (Peterson, 1991). Moreover, the search for a reasonable explanation of cause and effect has been further complicated by the recognition that a significant number of clinically well subjects also carry the presumptive infectious organism. Clearly, diagnostic tests directed 30 solely at *H. pylori* would not have a suitable specificity. Therefore, for these diseases and others related to an infectious agent, new approaches are needed.

35 It is known that microbial proteins may be antigenic, and possibly allergenic. But there has been no systematic pursuit of a set of individual antigenic molecules that derive from a microorganism and that highlight

interactions between the microorganism and a host to produce symptoms of a disease or condition.

Crude, impure antigen preparations based on size exclusion chromatography or detergent solubilization have 5 been prepared from *H. pylori* in attempts to monitor IgG or IgA responses or to develop crude vaccines. However, presence of IgG or IgA antibodies to *H. pylori* is not dispositive of an allergic reaction to an allergen, and therefore would not indicate allergen-based disease. 10 Also, no previously known assays utilize immunological profiles to facilitate preparation and administration of "customized" vaccines.

Measurements of total immunoglobulin, even of a certain type, is a relatively crude assay because it 15 measures a response to many antigens from many sources. Attempts to develop serological tests consisting of detecting antibodies in serum to crude extracts of bacteria have had unacceptably high false positive and false negative rates (Evans, 1989). Use of purified 20 antigens showed some improvement. At most, assays for one antigen or allergen, or for a crude composite of antigens or allergens, are available for clinical diagnosis, but are unsatisfactory. Multivariate approaches to define a set of individual antigens 25 specific for a microorganism, and to determine an immunological response with increased sensitivity and specificity have not been suggested.

It also is recognized that there are immunological responses of a host to the presence of a microorganism. 30 Additionally, only IgG and IgA isotypes, which are separate and distinct isotypes from IgE with quantitatively different serum levels and different patterns of response to infectious microorganisms, have been previously described in this context. For example, 35 a procedure for IgG determination of the presence of *H. pylori* in serum samples, uses as antigens *H. pylori* sonicates, whole cells and purified antigens. Sonicates are bacteria harvested in a buffer, sonicated and cleared

of cellular debris by centrifugation. Outer membrane suspensions were also used for oral immunization (Czinn & Nedrud, 1991). Yet immunological profiles have not been identified heretofore that are specific for 5 complexes between a set of individual microbial antigens and host serum antibodies which identify an organism associated with a disease or condition.

Attempts to provide vaccination against diseases related to microbial antigens have not been successful, 10 and the approaches taken are unlikely to be successful by virtue of their serious limitations. For example, some vaccines have been developed that entail the utilization of only one or two *H. pylori* proteins as immunogens. This is an insufficient number of immunogens because not 15 all subjects possess the ability to be immunized by only a few selected antigens. In other subjects whose immune systems have the ability to recognize these few antigens, the strength of immunization may be insufficient to provide either short term eradication and/or long-term 20 protection against re-infection. Attempts at vaccination have also failed to take into account that antigenic *H. pylori* proteins universally induce IgE reactivity among subjects, and in an unpredictable way. If a subject is immunized with any such proteins, the subject faces a 25 high likelihood of experiencing an anaphylactic reaction which can be immediately life-threatening. For example, auto-vaccination of a woman with antrum gastritis and *H. pylori* infection provoked an allergic reaction. (Varga et al., 1992).

30 Vaccination is a more desirable treatment than antibiotic therapy because vaccination is generally more effective, more specific, and is much less limited by the evolution of resistant microbial strains. For example, at present no antibiotic regimen is 100% effective in 35 eradicating *H. pylori* at any given therapeutic event (at best, antibiotics are 80-90% effective per infective episode). As stated by Czinn and Nedrud (1991), "a reliable method for long-term eradication of *H. pylori*

does not exist, . . . and at least two or three anti-microbial agents are necessary to achieve temporary eradication. Thus, once *H. pylori* infection is established, it is difficult to eradicate." Another 5 limitation of antibiotic therapy for diseases related to this microorganism is that *H. pylori* strains are expected to quickly evolve which are antibiotic-resistant to those drugs being used at present. Therefore, a constant stream of new antibiotics must be developed. Another 10 limitation is that antibiotics do not provide long-term protection from reinfection because they cannot be administered chronically.

15 Immunization of mice and ferrets with killed *H. pylori* induced IgA and IgG anti-*H. pylori* antibodies. In gnotobiotic piglets, parenteral vaccination with formalin-killed *H. pylori*, and oral vaccination with live bacteria induced *H. pylori*-specific serum immunoglobulins G, M and A. But vaccination did not prevent infection, and it did lead to increased severity and activity of 20 gastritis (Eaton and Krakowka, 1992). A program for effective immunization in humans still does not exist. It is this deficiency that is addressed in the present invention.

#### SUMMARY OF THE INVENTION

25 Pursuant to the present invention, a microorganism associated with a disease or condition is not directly detected. Instead, an immunological response profile of the infected host is detected which reflects reaction of the host to a library of individual antigens specific for 30 the presence of the microorganism.

35 All embodiments of the present invention utilize the novel concept of an antigen library for the determination of immunological profiles for monitoring the course of disease. Libraries are a plurality of antigens/allergens from the same species of microorganism purified to homogeneity. Individual molecules are characterized by molecular weight, isoelectric point, solubility and the

like. The present invention relates methods of characterizing an allergic response to a microorganism by monitoring an individual's IgE response. The allergic response is indicative of a disease state. Neither an IgA nor an IgG immune response is dispositive of an allergic reaction. The disease state can be ameliorated through administration of "customized" vaccines which make use of antigen/allergen libraries and the immunological profiles present in a person who is a candidate for vaccination.

The methodology of the present invention elevates the search for disease related antigens, particularly, antigens that stimulate an allergic response, from a "needle in a haystack" approach to one based on a sophisticated plan leading to detection of specific host invaders that produce clinical symptoms. Multivariable diagnostic criteria based on identification and quantification of immunoglobulins binding to a library of specific antigens are used to detect specific diseases or conditions and to differentiate them from related diseases or conditions.

The diagnostic methods disclosed herein have great flexibility because they are based on a library of microbial antigens from which a test vector is selected depending on the specificity-sensitivity levels desired. Because specificity and sensitivity are correlated, altering one value generally affects the others. Choice of a test vector follows assay optimization techniques wherein antigen groupings are mixed and matched to obtain the desired balance of sensitivity and specificity. Tests designed to increase sensitivity generally do so at the risk of lowering specificity. The present invention permits the modification of these values for particular situations. Because there are large numbers of antigens in a library, there are many subsets that may be generated for a particular assay. Also, because changes in IgE, IgA, IgG and IgM directed to the set of chosen antigens are monitored, comprehensive rather than "one

"shot" information is provided for an individual being tested.

By means of the present invention, an immunological response is detected that occurs in a host and is correlated with clinical suspicion of a disease, such as chronic gastritis and peptic ulcer, to arrive at a diagnosis. Discrimination between diseases with similar symptoms is facilitated by testing not for the response to only one antigen, but rather by testing for the response to a library of antigens determinative of the presence of a particular bacterium in a particular disease state or condition. This novel concept is extendable to disorders related to bacterially stimulated allergic responses, wherein detection of immunoglobulins such as IgE directed to protein subfractions of a bacteria, opens the door to a complex, multivariable approach to diagnostic assays, and exposes the mechanisms producing disease symptoms.

The methods of the present invention include chemically dissecting a microorganism such as a bacterium, a virus or a mycoplasma, into purified protein (antigenic) subfractions, each terminal subfraction containing an individual molecule capable of eliciting an immunological response in a host. The identifying subfractions are produced by dissecting a microorganism so finely into its individual molecular components, that subfractions or a combination thereof which include the constituent molecules are produced that uniquely identify the microorganism. Generation of purified antigens also enhances binding of immunoglobulins to a specific antigen because specific antigen absorption sites or coupling sites on a test surface are not cluttered with contaminating, non-specific antigens. Using a library of antigens also takes advantage of the commonality of protein molecules among different strains of a species.

A method for isolating and identifying individual microbial antigenic proteins includes the steps of treating the microorganism, preferably a bacterium, with

increasing concentrations of sodium dodecyl sulfate (SDS) and precipitating proteins (polypeptides) within each SDS preparation with increasing concentrations of acetone. Polyacrylamide gel electrophoresis is used to further 5 separate the polypeptides by molecular weight. By this process, an individual molecule is isolated and may subsequently be visualized by labelling bands on the gel, for example. A library (protein bank) of such proteins is generated from a particular species of microorganism, 10 such as those enumerated in Table 1 below. An "individual molecule" is a single species as identified by molecular weight, isoelectric point, solubility and the like. The purification methods of the present invention produce in the terminal subfractions, that is 15 after the last acetone treatments, individual molecules.

In accordance with another embodiment of the present invention, preparing purified protein antigens, which can also be allergens, can be accomplished by (a) treating bacterium containing a protein allergen with acetone to 20 remove lipid components; (b) disrupting the acetone-treated bacterium in a solution comprised of buffer, salt, metal chelator, protease inhibitor, and benazamidine; (c) separating a protein containing fraction from complex carbohydrates and nucleic acids; 25 (d) collecting a composition comprised of proteins which are of molecular weight at least about 1,000; and (e) separating the proteins of the composition of (d) by ion-exchange chromatography. This embodiment does not yield antigens as pure, however, as those produced by the SDS 30 acetone method described in a previous paragraph. Under standard assay conditions, the purer the microbial antigen, the larger are the number of specific antigen sites available for binding with antigen specific immunoglobulin. An advantage of the purified antigens is 35 that they are detectable by one immunoglobulin isotype in the presence of other isotypes.

By use of the SDS-acetone method, a library of antigens is derived from fractionating a microorganism

into individual molecules identified as bands of a uniform molecular weight, and determining that each individual molecule is capable of complexing with an immunoglobulin. From that initial library, subsets are 5 selected for different purposes.

To facilitate collection of bacterial antigens in a quantity adequate for use in diagnosis and treatment, production of the antigens by recombinant genetic technology is preferred.

10 Each purified immunogenic antigen is sequenced by techniques known to the art. Partial sequences of the COOH and NH<sub>2</sub> ends of each antigen are determined. From this information recombinant DNA methodologies evolve such as cloning genes and developing primers for use in 15 PCR (polymerase chain reactions). Because the organisms that are the source of the antigens are simple, generally there will be only one gene coding each antigen. Each sequenced protein is expressed in an efficient cell culture production system such as in *E. coli* or Chinese 20 hamster ovary (CHO) cells to provide sufficiently large quantities of purified antigen for use in diagnostic or screening assays or vaccine manufacture.

An antigen mapping serum assay system is employed to 25 screen patient sera rapidly and efficiently for IgA, IgE, IgG and IgM reactivity toward each *H. pylori* antigen in a disease-specific library. If the library is to be used for screening purposes in an assay in which the immunoglobulin response will be detected in the aggregate, a large enough library is selected to generate 30 a detectable signal, for example on a paper disc. For this purpose, antigens derived from a particular microorganism that are not unique to that microorganism, may be included.

Because not all patients may react with the same 35 subset of antigens within the basic library, enough antigens are included in the assay so that a detectable signal is generated from all patients having a particular condition. This means a positive test (that is, a signal

detectable at a predetermined level) may differ in its antigenic composition for patients having the same condition.

5 The number of antigens required to detect a condition is a function of specificity and sensitivity levels desired, and the labelling method used. For example, if it is more important not to include non-affected individuals as false positives, that is, if a high specificity is desired, then a relatively smaller subset 10 of highly specific antigens from the library is selected.

15 But the largest subset of antigens from a library generally will provide optimum sensitivity and specificity. Sensitivity is improved because enough pure antigens are provided to be detected by one isotype in the presence of other specifically-reactive antibodies of other isotypes. To further enhance sensitivity of IgE based detection, "scrubbing" serum samples to be tested is performed. "Scrubbing" is a procedure by which specific immunoglobulin isotypes are absorbed from a 20 serum sample. The preferred procedure is to absorb all IgG from the sample. Removing IgG, IgA, and IgM, or both IgG and IgA, or both IgA and IgM, or IgG and IgM, or only IgA or only IgM, is also helpful. Assays based on a single antigen may have poor sensitivity, although they 25 may be highly specific because not all patients may be sensitive to a particular antigen, or the antigen is not expressed by all subspecies of a particular microorganism. The use of a family of antigens in the present invention is a solution to the problem.

30 In some diseases or conditions, more than one microorganism may be implicated in causality of a disease by relatively crude analysis such as culturing organisms from a clinical sample. Not all of these microorganisms may prove to be specific for a disease or condition, as 35 determined by methods of the present invention. Comparison of immunological profiles of individuals in response to antigen libraries prepared from the different microorganisms suspected of association with the

condition, may reveal the microorganism primarily responsible for characteristic symptoms, and may differ from incidental agents. An organism that is incidental will not show an IgE response, or will not show as strong an IgE response, as an organism operating as an allergen provider responsible for the condition.

The immunological response of each protein library (protein bank) is tested for a specific immunoglobulin reactivity. A profile is then developed of antigens eliciting a response from persons having a particular disease or condition.

A profile is defined as an immunological response to a set of specific individual antigenic molecules isolated in subfractions prepared from a microorganism. The ability of the antigens to discriminate between persons with and without a condition is determined by the specificity of the immunological response to a library of antigens in a sample from a person with a disease or condition, as compared with a control sample. An adequate control is defined, depending on the condition to be identified. Adequate controls include individuals without the condition, without clinical symptoms of a disease, or with a disease or condition in which a differential diagnosis is desired. Controls are ideally matched or standardized for variables known to stimulate an immunological response. Immunological profiles are comprised of the types of immunoglobulin produced and the amount of each type produced. Immunoglobulins suitable for the practice of the invention include IgA, IgM, IgG and IgE.

IgE is preferred as the immunoglobulin used in the initial assay because positive values indicate the presence of an allergen, and because IgE responses are more characteristic of the conditions to be detected within the scope of the present invention.

An IgE response usually indicates chronic or protracted exposure to an antigen and requires a longer exposure to evolve, compared to the other immunoglobulins

such as IgA, IgG or IgM which arise relatively quickly in the presence of an antigen.

After the IgE-mediated response is detected, however, monitoring the condition, particularly as to a response to treatment, is preferably accomplished by ascertaining IgA, IgG and/or IgM levels. For example, detectable anti-*H. pylori* IgG antibodies develop long after acute infection and persist as long as infection is present. A vector of responses for the immunoglobulin types reveals more than values for one immunoglobulin alone.

10 A profile specific for a microorganism is identified by binding of an immunoglobulin isotype to a library of antigenic subfractions of the microorganism associated with the disease.

15 An individual molecule or a combination thereof is detected by measuring the immunological response of the host in the presence of the subfraction containing the molecule. Although in the host, the subfraction is generally part of an intact microorganism.

20 Microbial specific protein subfractions are identified by measurement of serum immunoglobulin levels, IgE, IgM, IgG and IgA, indicating that microbial specific immunoglobulin is quantifiable. IgE response is the preferred initial screening assay because reaction with 25 this immunoglobulin isotype is more specific for an antigen library. An IgE response generally indicates chronic exposure to an antigen, and requires a longer exposure to develop. The other immunoglobulins arise more quickly and are more likely to reflect random or 30 sporadic, rather than causal, exposure. After the IgE response is detected, reactions with IgA, IgG and IgM are useful in addition to monitor responses of individuals to treatment and/or disease progression.

**Table 1: Method of Isolating and Identifying  
Individual, IgE-Reactive Bacterial Proteins**

5

**Solubilization and Fractionation  
of Bacterial Proteins**

10

**10 Distinct Acetone-Precipitated Protein Fractions**

**Each Acetone-Precipitated Protein Fraction  
Further Subfractionated by Polyacrylamide Gel Electrophoresis.  
Individual Protein Molecules Visualized.  
A Protein Library (Bank) is Defined**

15

**Compare Potential IgE-Reactivity of Each Protein Library (Bank) by  
Western Blot Analysis Using Patient and Control Sera**

**Those IgE-Reactive Bands of the Blot Highlighted with Patient (e.g., Ulcer  
Patients that are Colonized by *H. pylori*) Sera But Not Control  
Sera are Indicative of Allergenic Proteins that are Highly  
Specific for the Bacterial Species to be Analyzed.**

Another aspect of the present invention is a method of determining whether an individual has an immunological response to a bacterial antigen, the method including  
5 (a) providing serum from an individual suspected of containing the immunoglobulin directed to the antigens of the agent; (b) providing a composition consisting essentially of a purified specific antigenic library;  
10 (c) reacting the serum of (a) with the composition of (b) under conditions which allow immunological binding between an antibody and an antigen to which it is directed; and (d) detecting complexes formed, if any, between antibodies in the serum of (a) and each of the individual protein antigens in the composition of (b).

Accordingly, the present invention contemplates a  
15 method of measuring IgE which bind(s) immunologically to an allergenic protein(s). Serum suspected of containing the IgE is reacted with an extract of the microorganism coupled to a solid support, followed by washing and reacting with labelled anti-IgE, and detecting labeled  
20 anti-IgE bound to the solid support.

A suitable method of identifying allergic immunological responses is to couple one or more allergenic proteins (polypeptides) which include epitopes to a solid substrate. A biological sample, such as serum  
25 or tissue fluids, suspected of containing IgE specific for the allergens, is reacted with the allergen-substrate complex. IgE that reacts immunologically with the allergen of the complex is detected by methods such as Western Blots and ELISA (enzyme-linked immunosorbent assay). Because anti-isotype antibodies are available, the immunoglobulin may be identified and quantified without separating them. Enhanced sensitivity is a  
30 result of providing enough pure antigen so that even if more than one isotype of antigen specific immunoglobulin binds to the antigen, each is detectable separately.

The reason that the effect of each isotype is detectable against a background of the other isotypes, is that there is sufficient antigen available so that

binding sites are available to accommodate specifically-reactive immunoglobulins of all isotypes. Competition for sites does not dilute binding of an isotype such that label detection of each isotype is obscured.

5 An example of an assay which is suitable for detecting IgE directly to crude antigen extracts is the Radioallergosorbent (RAST) test. In a modified RAST test, purified protein allergens are linked to a solid support.

10 If the proteins are not purified enough so that sufficient sites are available for binding of all isotypes to a degree that the binding is detectable, prior to reaction with the protein allergens, the serum to be tested is treated to remove IgA, IgM and/or IgG. 15 This "scrubbing" step is suitable for the detection of the allergen-specific IgE. "Scrubbing" is not required for a RAST test if the purified antigens of the present invention from the SDS-acetone method are used in sufficient quantities, although in some instances it may 20 enhance sensitivity.

Via an illustrative embodiment of the present invention which employed a modified RAST test, it was discovered for the first time that there was a high positive correlation between gastritis/ulcer disease and 25 the presence of IgE directed to specific subfractions of protein allergens of *H. pylori*. These results provided direct evidence, for the first time, that an adverse immune reaction to the antigens of this bacterial species is responsible for the pathological reaction in the host, 30 in particular, as evidenced by the existence of a hypersensitivity reaction mediated by specific IgE components.

35 *Helicobacter pylori* has been identified as a principal cause of chronic gastroduodenal disease (chronic gastritis and peptic ulcer disease). However, recent epidemiologic studies indicate that many asymptomatic subjects with no overt disease may harbor gastroenteric *H. pylori*. Therefore, additional factors

may determine whether *H. pylori* carriers develop disease. A principal factor may be a genetically predicated, localized *H. pylori*-specific IgE-mediated immune response. The existence of such a response is documented. The specific IgE reactivity is targeted against a large library of *H. pylori* protein antigens and appears to be equally prevalent among disease-positive subjects from southeast China, Hungary and the U.S. Significantly fewer healthy subjects who are shown to be *H. pylori* carriers by specific IgG serology are IgE-positive. This suggests that IgE-mediated inflammation may be a differentiating factor in determining which *H. pylori* carriers develop gastroduodenal disease.

The identification of protein allergens of *H. pylori* associated with gastritis/ulcer disease allows for a relatively non-invasive detection of the disease. In addition, it also allows for treatment of the disease by immunotherapy, using purified protein allergens.

Investigation of bacterium-specific IgE fractions is not limited to diseases associated with *H. pylori*. in addition, immunoglobulins other than IgE (IgA, IgG, IgM) are suitable for the practice of aspects of this invention.

Another aspect of the invention is a composition consisting essentially of a purified antigenic subfraction prepared from a microorganism by the methods of the present invention. Specifically, the subfraction or combination thereof includes at least two bacterial antigens. More specifically, subfractions are derived from *Helicobacter*, *Pseudomonas*, *Streptococcus* and the like.

Another aspect of the invention is a set of protein antigens (library, protein bank) coupled to a solid substrate. The set includes antigens specific for *H. pylori*.

A "set" (library, vector, protein bank) of antigens is defined as polypeptides that invoke an immunological response and distinguish a biological sample from an

individual with a condition, to a sample from an individual without the condition.

In an illustrative embodiment, comparison of serum from individuals affected with a condition or disease, to 5 serum from control (unaffected) individuals shows the power of such a procedure for isolating and identifying individual, IgE-reactive bacterial proteins. IgE-related molecular bands on a solid support that are highlighted and are present in serum from the affected, but not from 10 control samples, are highly diagnostic for a disease or condition, for example, *H. pylori* and peptic ulcer in Tables 3 and 4. The problem of false positives in well persons using a direct assay for *H. pylori* is alleviated because only an *H. pylori*-stimulated response is scored 15 as positive.

It was unexpected that antibodies produced to the subfractions containing isolated and purified bacterial antigens are capable of teasing out subtle differences in the antigenic components of even closely related species 20 or differences in allergic responses of the host. It was also unexpected that a signal signifying antigen-antibody binding to one immunoglobulin isotype is detectable in the presence of others.

Moreover, even in situations where the presence of 25 an infectious agent does not discriminate between the presence or absence of a disease, the severity of a disease may be determined by quantifying the immunoglobulin response to an antigenic profile of the agent.

30 The problem of false positives using currently known or available assays for *H. pylori* in well persons is alleviated because only an *H. pylori*-specific response is scored as positive.

Still another aspect of the invention is an 35 immunotherapeutic method of treating an individual for a disease resulting from an allergic reaction to a bacterial infection. The method includes the steps of introducing into the individual a composition consisting

essentially of a subfraction of antigens from the bacteria, including both specific and non-specific antigens, wherein the conditions of the introduction are sufficient to alleviate the symptoms of the allergic reaction. The detailed information obtained from the methods of the present invention enable treatment compositions to be rationally designed rather than designed merely by trial and error.

As an example of this aspect of the invention, a method of treating an individual for a disease associated with a microorganism as defined herein, if *H. pylori* induced gastritis is the disease to be treated, is to prepare a composition comprised of a polypeptide which contains one or more epitopes that are immunologically identifiable with immunogenic epitopes of *H. pylori*. The polypeptide is delivered to the individual to be treated in an amount sufficient to relieve an allergic reaction to *H. pylori* in the individual. The treatment composition is further comprised of a suitable excipient and is introduced into a patient.

Still another aspect of the present invention is a diagnostic kit including a library of microbial antigens that specifically identify a microorganism. The antigen library is packaged in a suitable container. This library includes a polypeptide containing at least one epitope which is immunologically identifiable as a microbial epitope. The antigen is affixed to a solid support. The kit also includes means for detecting immunological complexes formed between the antigens and an immunoglobulin in the biological sample. Detecting means include use of a radionuclide, radiolabel, fluorophor, chemiluminescent molecule or an enzyme, or other easily detectable labels.

Yet another aspect of the invention is a composition comprised of a structural analog of an epitope of a bacterial antigen, wherein the structural analog binds to an immunological paratope.

Another aspect of the invention is a composition comprised of a purified polyclonal antibody directed to a microbial antigen of the present invention.

5 Yet another aspect of the invention is a composition comprised of a monoclonal antibody directed to an antigen of a microorganism of the present invention.

Because infection may be asymptomatic until serious complications have occurred, vaccination is preferred to prevent late, life-threatening effects. The vaccines of 10 the present invention entail the use of many available immunogens selected from a large antigen library in order to provide wide spectrum antigen coverage. By providing a multi-antigen vaccine, all immunized subjects can attain a sufficiently strong immune response which also 15 is more likely to be effective long-term. By ruling out toxicity (anaphalaxis) of the antigens, selecting those generating an immune response, and combining the responses to the multiple antigens, a structure analogous to a strong rope is built from many individually less 20 strong strands, wherein each strand is a response to an individual antigen.

Use of a "customized" vaccine diminishes the risk of anaphylaxis because an immunological profile is generated for each prospective patient that delineates the 25 individual's immune response, categorized by isotype, to each antigen of the library. Antigens which have already elicited an IgE response in the individual are not included in the "customized" vaccine. Thus, antigens included in the vaccine may eventually provoke an IgE 30 response, but the response will not result in anaphylaxis because antigens that have previously elicited an IgE response in that individual will not be included in the "customized" vaccine.

An additional consideration in the formulation of a 35 "customized" vaccine is minimization of induction of an auto-immune response. If sequence information is available for the antigens comprising the library, it is compared with amino acid sequences of other human

proteins via computer-aided protein database comparison. Antigens that demonstrate substantial identity in their epitopic regions with human proteins are not included in a customized vaccine. In the absence of epitopic 5 sequences or in the absence of sequence information for the antigens comprising the library, cross-reactivity of antigens is used to eliminate an auto-immune response. Antigen cross-reactivity can be determined through a variety of techniques, such as the Ouchterlony method of 10 double diffusion.

The goal of vaccination is to increase the humoral response (i.e., the IgA or IgG response) to disease associated-microorganisms, and also to increase cellular immunity. To develop a customized vaccine from the 15 antigen library of the present invention the following steps are taken:

- (1) Screening each subject to be vaccinated for the presence of serum IgE specific for any of the proteins in the antigen library of the present 20 invention.
- (2) Not using as immunogens any IgE-reactive proteins for the respective subject because of high anaphylaxis risk;
- (3) Not using proteins or segments of proteins that are 25 homologous to human proteins and that could act as auto antigens;
- (4) Screening the remaining proteins for serum IgA and IgG reactivity. Among subjects known to be carrying *H. pylori*, preference is given toward including in 30 a vaccine those antigens which are already inducing a significant IgA and/or IgG humoral response. Because an immune response is desired that favors gastroenteric clearing of the infectious bacteria, those proteins already eliciting an IgA response may 35 be more useful in the vaccination process than those not eliciting such a response.

In addition to a humoral response, induction of a cellular response is useful as part of the antigen

selection process. Overall criteria for a subset of antigens that elicits an optional humoral and/or cellular response are those recognized as clinically significant by those of skill in the art.

5        The antigenic *H. pylori* proteins are administered in any number of ways known in the art in order to effect vaccination. Either oral or parenteral routes of administration are contemplated. One such method entails administration of the desirable antigens along with a 10 standard adjuvant. Another method uses a library of altered vaccinia viruses, each virus possessing one of the antigens found in the *H. pylori* antigen library. After ruling out those vaccinia strains carrying the expressed IgE-reactive proteins, the remainder of the 15 viruses are used in a preferred mixture for immunization without the need to use adjuvants for the immunization process.

Preparation of customized vaccines entails a preliminary identification of those antigens which may be 20 useful as vaccine components but which are also IgE-reactive in the individual subject. These antigens are kept out of those selected for the vaccines.

The vaccine process of the present invention entails formulation of the best possible combination of 25 immunogenic proteins for each individual subject. This provides the most ideal combination of antigens from a proprietary antigen library while minimizing the risk of anaphylactic reaction to any single antigen.

30        The following terms are employed in this description with the follow meanings:

allergen refers to an antigen that gives rise to allergic sensitization by IgE antibodies.

35        allergoid refers to a chemically modified allergen that gives rise to antibody of the IgG but not IgE class, thereby reducing allergic symptoms.

allergy denotes an altered state of immune reactivity, usually denoting hypersensitivity.

5                   **antibody** refers to a polypeptide or group of polypeptides which are comprised of at least one antibody combining site. An "antibody combining site, or "binding domain", is formed from the folding of variable domains of an antibody molecule(s) to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows an immunological reaction with the antigen. An antibody combining site may be 10 formed from a heavy and/or a light chain domain (VH and VL, respectively), which form hypervariable loops which contribute to antigen binding. A "paratope" is an antibody-combining site for an epitope, the simplest form of an antigenic determinant. The term "antibody" 15 includes, for example, vertebrate antibodies, hybrid antibodies, chimeric antibodies, altered antibodies, univalent antibodies, the Fab proteins, and single domain antibodies.

20                   **antigen** is a substance capable of generating an immune response recognized by T- and/or B-cell proteins and in the present invention the term is limited to polypeptides.

25                   **biological sample** refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents.

30                   **coupled** refers to attachment by covalent bonds or by strong non-covalent interactions (e.g., hydrophobic interactions, hydrogen bonds, etc.). Covalent bonds may be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like.

35                   **epitope** refers to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope.

Generally an epitope consists of at least 5 such amino acids, and more usually, consists of at least 8-10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and 5 include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance.

10 **immunogenic** refers to an agent used to stimulate the immune system of a living organism, so that one or more functions of the immune system are increased and directed towards the immunogenic agent.

**immunogenic polypeptide** is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant.

15 **immunologically identifiable with/as** refers to the presence of epitope(s) and polypeptides(s) which are also present in the designated polypeptide(s). Immunological identity may be determined by antibody binding and/or competition in binding; these techniques 20 are known to those of average skill in the art, and are also illustrated infra.

25 **immunoreactive** refers to a polypeptide when it is "immunologically reactive" with an antibody, i.e., when it binds to an antibody due to antibody recognition of a specific epitope contained within the polypeptide. Immunological reactivity may be determined by antibody binding, more particularly by the kinetics of antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an 30 epitope against which the antibody is directed. The techniques for determining whether a polypeptide is immunologically reactive with an antibody are known in the art. An "immunoreactive" polypeptide may also be "immunogenic."

35 **individual** refers to a vertebrate, particularly members of the mammalian species, and includes, but is not limited to, domestic animals, animals used for sport, and primates, including humans.

**label** refers to any atom or moiety which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a polynucleotide or polypeptide.

5       **polypeptide** refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the 10 polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid, including unnatural 15 amino acids, for example, polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. The term "polypeptide" does not connote the method by which the molecule was made, and thus includes naturally occurring molecules, as well as molecules made 20 by chemical or recombinant synthesis.

25       **support** refers to any solid or semisolid surface to which a desired polypeptide. Suitable supports include glass, plastic, metal, polymer gels, and the like, and may take the form of beads, wells, dipsticks, membranes, and the like.

30       **treatment** refers to prophylaxis and/or therapy.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graph showing the effect of scrubbing serum with Protein A on the detection of anti-*H. pylori* IgE in a modified RAST test.

35       FIGURE 2A is a graph showing the serum IgE levels of IgE directed to subfractions of *H. pylori* protein allergens in healthy individuals (controls).

FIGURE 2B is a graph showing the serum IgE levels of IgE directed to subfractions of *H. pylori* protein allergens in gastritis patients.

FIGURE 3 is a plot of the net total IgE immunological reactivity of serum from control and gastritis patients using all available *H. pylori* protein fractions isolated from an HPLC DEAE column; patients' values are in column 5 1, and control values are in column 2.

FIGURE 4 is a plot of the net total IgE immunological reactivity of serum from control and gastritis patients with the proteins in fractions 59, 64, 66, 68, 72 and 74 of the HPLC DEAE column.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to preparation of customized vaccines for diseases related to antigens/allergens of microorganisms. The vaccines are prepared from antigens/allergens in a library derived from microorganisms. The library includes "individual molecules" that are a homogeneous species identified by molecular weight and/or isoelectric properties and solubility. In particular, the antigens/allergens used according to the present invention are derived from microorganisms associated with diseases and conditions for which diagnosis and treatment are needed. In the present disclosure, when the term "microorganism" is used, it is used to encompass microorganisms that are suitable for the present invention, such as bacterium, virus, mycoplasma and the like.

The antigens/allergens (used interchangeably with polypeptides or proteins herein) are extracted from preparations of a microorganism, and subfractionated in a hierachal fashion until individual protein molecules are each in a separate subfraction, as defined by molecular weight. A preferred protocol is shown in Table 2 for the solubilization and fractionation steps. The protocol may be extended or modified in purifying antigens from a specific microorganism until individual molecules are present in the terminal subfractions and visualized on a polyacrylamide gel or other materials which separate molecules by molecular weight.

A library of antigens is selected that is specific for a particular disease or condition, by determining a set of antigens that evoke immunologic responses in percentages of individuals with the disease or condition, that are higher than in control individuals. For some embodiments, a set of antigens are selected that are unique to a microorganism in a condition as compared to a control.

The methods described here employ one or more polypeptides which contain one or more bacterial epitopes

which form antigen-antibody complexes with immunoglobulins directed to bacterial antigens. To detect and quantify Ig response to bacterial allergens, for example, a Western Blot analysis or a modified RAST test as described below is suitable. For analysis of IgG, IgM or IgA response, an ELISA is suitable.

Methods of the present invention are useful for the diagnosis, treatment and prevention of microbial related diseases. In an illustrative embodiment, percent-positive prevalence of serum IgE reactivity in peptic ulcer patients versus nasal polyp patients is shown for differential *H. pylori* antigens in Table 3. Thirty-one antigens separated into two molecular weight categories, with 50 kD as a division, are identified and listed in this Table. Eleven peptic ulcer patients and 20 nasal polyp patients were selected by direct clinical examination and, in all cases, by laboratory documentation of *H. pylori* in the ulcer patients. IgE was determined by the method of Western Blot as described herein. As illustrated in Table 3, the library of *H. pylori* antigens in this comparison, discriminates between individuals with the two diseases. It also is evident that some antigens are present in higher percentages of ulcer patients than other antigens.

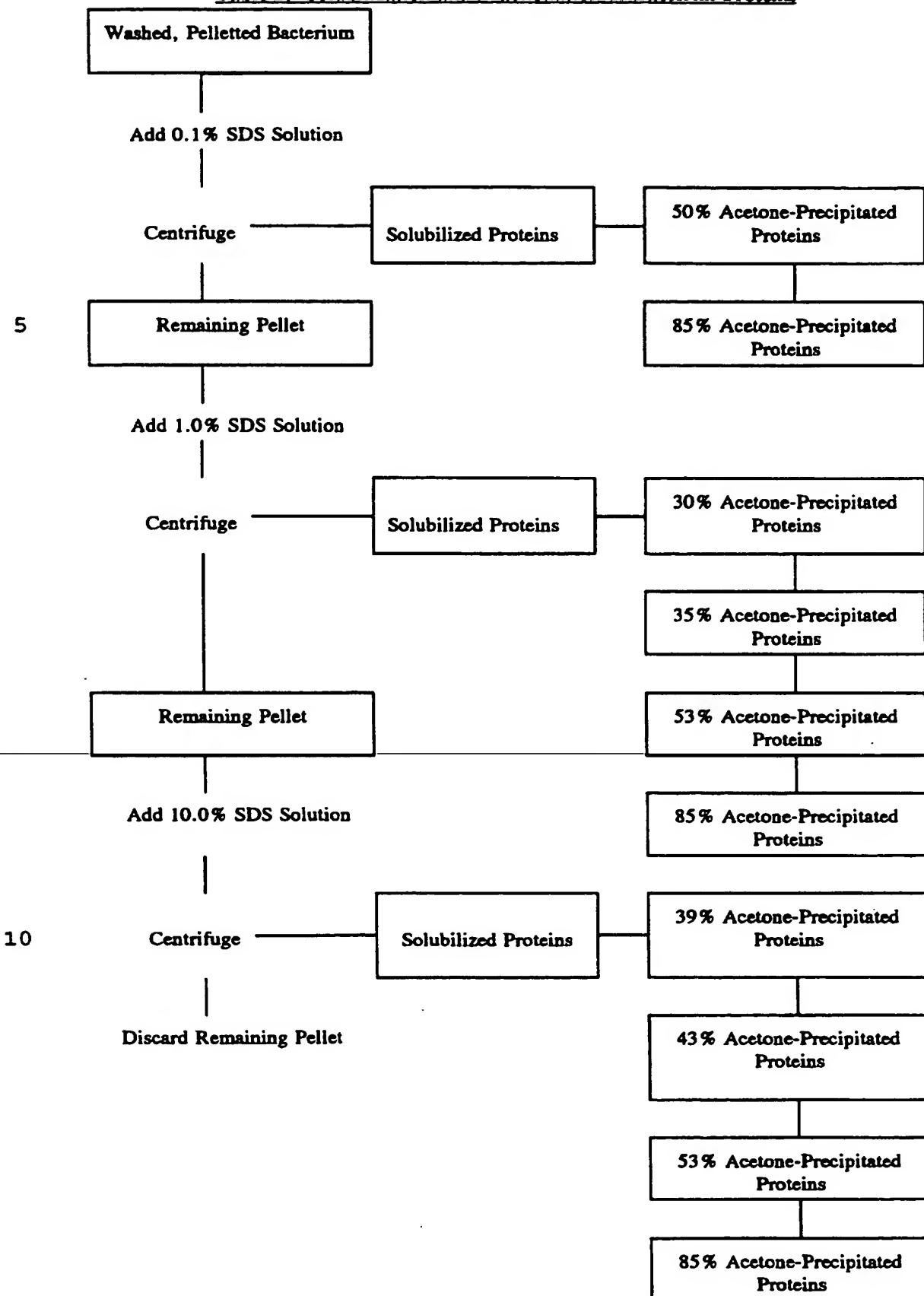
Table 1 illustrates steps leading to determining how a group of bacterial antigens are defined as a "library." A library is defined as a set of antigens that react immunologically with at least some of the affected individuals. In some embodiments, it is preferable to select all positive antigens to enhance the signal generated by immunological complexes. In other embodiments it is preferable to define as a library, a set of antigens that only reacts with a large percent of affected individuals. Although some antigens in the library may not be completely specific, in the aggregate, their effect will be minimal on test accuracy because their non-specificity will be diluted and masked, they

will be diluted by the effects of the other specific antigens.

After the antigenic polypeptides are isolated and purified, they are sequenced and used to develop 5 recombinant genetic vectors which are capable of expressing the polypeptides in a host such as *E. coli*. These methods are disclosed in a subsequent section and are useful for producing large quantities of antigens.

10 Table 4 lists antigens designated by identification numbers (1.12.1 and the like), by molecular weight (48 and the like), and sources (polyp 1, and the like). A "+" indicates a positive immunological response (binding with IgE), a "-" indicates no response.

15 The present invention further comprehends, *inter alia*, (i) methods to test for an immunological response of a host to a library of bacterial antigens, (ii) diagnostic kits, (iii) methods for treating diseases found to be associated with a specific immunological profile, as defined here, (iv) vaccines, (v) antibodies 20 to the bacterial antigens detected by the methods described here and (vi) methods of producing antigens using recombinant genetic technology.

**Table 2: Solubilization and Fractionation of Bacterial Proteins**

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Table 3: Percent-Positive Prevalence of Serum IgE Reactivity of Differential *H. Pylori* Antigens

	Antigens > 50 kD	Peptic Ulcer Patients		Nasal Polyp Patients	
5	1) 2.7.1		55%		5%
	2) 2.7.2		36		0
	3) 4.7.1		9		0
	5) 7.7.3		36		5
<hr/> Antigens < 50 kD <hr/>					
10	6) 1.12.1		9		0
	7) 1.12.2		9		0
	8) 2.12.1		18		5
	9) 2.12.2		27		0
	10) 3.12.1		9		0
15	11) 3.12.2		45		5
	12) 3.12.3		36		0
	13) 3.12.4		9		0
	14) 3.12.5		27		0
20	15) 3.12.6		36		0
	16) 4.12.3		9		0
	17) 4.12.4		9		0
	18) 5.12.1		9		0
25	19) 5.12.2		27		5
	20) 5.12.4		18		0
	21) 5.12.5		9		0
	22) 7.12.1		45		5
30	23) 7.12.4		9		0
	24) 8.12.2		36		10
	25) 8.12.4		9		0
	26) 8.12.5		18		0
	27) 9.12.1		18		0
	28) 9.12.2		18		0
	29) 9.12.3		9		0
	30) 10.12.1		9		0
	31) 10.12.4		9		0

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Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	8010	8011	8012	8013	8014	8015	8016	8017	8018	8019	8020	8021	8022	8023	8024	8025	8026	8027	8028	8029	8030	8031	8032	8033	8034	8035	8036	8037	8038	8039	8040	8041	8042	8043	8044	8045	8046	8047	8048	8049	8050	8051	8052	8053	8054	8055	8056	8057	8058	8059	8060	8061	8062	8063	8064	8065	8066	8067	8068	8069	8070	8071	8072	8073	8074	8075	8076	8077	8078	8079	8080	8081	8082	8083	8084	8085	8086	8087	8088	8089	8090	8091	8092	8093	8094	8095	8096	8097	8098	8099	80100	80101	80102	80103	80104	80105	80106	80107	80108	80109	80110	80111	80112	80113	80114	80115	80116	80117	80118	80119	80120	80121	80122	80123	80124	80125	80126	80127	80128	80129	80130	80131	80132	80133	80134	80135	80136	80137	80138	80139	80140	80141	80142	80143	80144	80145	80146	80147	80148	80149	80150	80151	80152	80153	80154	80155	80156	80157	80158	80159	80160	80161	80162	80163	80164	80165	80166	80167	80168	80169	80170	80171	80172	80173	80174	80175	80176	80177	80178	80179	80180	80181	80182	80183	80184	80185	80186	80187	80188	80189	80190	80191	80192	80193	80194	80195	80196	80197	80198	80199	80200	80201	80202	80203	80204	80205	80206	80207	80208	80209	80210	80211	80212	80213	80214	80215	80216	80217	80218	80219	80220	80221	80222	80223	80224	80225	80226	80227	80228	80229	80230	80231	80232	80233	80234	80235	80236	80237	80238	80239	80240	80241	80242	80243	80244	80245	80246	80247	80248	80249	80250	80251	80252	80253	80254	80255	80256	80257	80258	80259	80260	80261	80262	80263	80264	80265	80266	80267	80268	80269	80270	80271	80272	80273	80274	80275	80276	80277	80278	80279	80280	80281	80282	80283	80284	80285	80286	80287	80288	80289	80290	80291	80292	80293	80294	80295	80296	80297	80298	80299	80300	80301	80302	80303	80304	80305	80306	80307	80308	80309	80310	80311	80312	80313	80314	80315	80316	80317	80318	80319	80320	80321	80322	80323	80324	80325	80326	80327	80328	80329	80330	80331	80332	80333	80334	80335	80336	80337	80338	80339	80340	80341	80342	80343	80344	80345	80346	80347	80348	80349	80350	80351	80352	80353	80354	80355	80356	80357	80358	80359	80360	80361	80362	80363	80364	80365	80366	80367	80368	80369	80370	80371	80372	80373	80374	80375	80376	80377	80378	80379	80380	80381	80382	80383	80384	80385	80386	80387	80388	80389	80390	80391	80392	80393	80394	80395	80396	80397	80398	80399	80400	80401	80402	80403	80404	80405	80406	80407	80408	80409	80410	80411	80412	80413	80414	80415	80416	80417	80418	80419	80420	80421	80422	80423	80424	80425	80426	80427	80428	80429	80430	80431	80432	80433	80434	80435	80436	80437	80438	80439	80440	80441	80442	80443	80444	80445	80446	80447	80448	80449	80450	80451	80452	80453	80454	80455	80456	80457	80458	80459	80460	80461	80462	80463	80464	80465	80466	80467	80468	80469	80470	80471	80472	80473	80474	80475	80476	80477	80478	80479	80480	80481	80482	80483	80484	80485	80486	80487	80488	80489	80490	80491	80492	80493	80494	80495	80496	80497	80498	80499	80500	80501	80502	80503	80504	80505	8

Immunological responses may be assayed by Western Blot analysis (see Materials and Methods) or ELISA. These techniques are known to those of skill in the art (also see "MATERIALS AND METHODS" herein). Because anti-  
5 immunoglobulin antisera are available, immunoglobulin profiles for IgE, IgA, IgM and/or IgG reactive to the same library of antigens may be examined separately. There is no need to separate the immunoglobulin isotypes present in a biological sample before testing it. The  
10 reason the effect of each isotype is detectable against a background of the other isotypes, is that there is sufficient antigen available so that binding sites are available to accommodate immunoglobulin of all types. Competition for sites does not dilute binding of an  
15 isotype such that label detection of each isotype is obscured. However, to increase sensitivity, absorption of IgA or IgG or IgM or any combination thereof in a serum ("scrubbing") is useful. See Example 5 below.

Polypeptides containing one or more epitopes  
20 immunologically identifiable with epitopes of the antigens defined herein including recombinantly or synthetically produced polypeptides and allergoids are useful in the diagnosis of diseases, and for treatment of these diseases, in accordance with the present invention.

25 These polypeptides also are useful for the production of antibodies, both purified polyclonal and monoclonal antibodies, directed towards microbial epitopes. The antibodies in turn are useful in the purification of polypeptides that are isolated in accordance with the  
30 present invention. In particular, monoclonal antibodies are useful for the detection of antigens containing specific epitopes and may also be useful in the production of vaccines for diseases associated with microorganisms of the present invention.

Diagnostic Kits for Diseases or Conditions Associated with a Microorganism

5 Polypeptides including two or more epitopes from a specific antigen library which are immunologically identifiable with epitopes of bacterial antigens are packaged in diagnostic kits. The kits are used to test a biological sample from an individual to determine if a condition is present in the individual. Diagnostic kits include the polypeptides in suitable containers and kits 10 also include a means for detecting immunological complexes formed between the polypeptide and immunoglobulin in the biological sample, if any. Detection means include a radionuclide, radiolabel, fluorophor, chemiluminescent molecule, enzyme, or other 15 easily detectable labels. In some cases, the polypeptides are affixed to a solid substrate such as a paper disc, or polystyrene wells. The kit also contains other suitably packaged reagents and materials needed for the particular diagnostic protocol, for example, 20 standards, buffers, as well as instructions for conducting the test using the kit ingredients. Kits are also useful for quantifying and monitoring an immunological response. Control specimens are optionally included.

25 For general screening, kits preferably include as many antigens from a library as will invoke a detectable immunological response when the disease or condition is present. In other words, for screening, sensitivity should be high to detect all affected individuals, even 30 at the expense of lower specificity. False positives can be selected out with a second level test based either on a more specific vector of antigens, perhaps a vector unique to a microorganism, by examining the pattern of responses to the individual specific antigens rather than 35 to the aggregate response to a library, and/or quantifying the overall immunological profile reactive to a more specific set by measuring several immunoglobulin

isotypes. For screening, IgE is preferred; for monitoring IgA, IgM and IgG are added to the assay.

Treatment of Disease Associated with Bacterial Allergens

In another embodiment of the invention, individuals suspected of having a propensity for, or who are affected with, a bacterially induced disease are treated with substances which reduce the allergic response to the microorganism. The goal of this treatment is antigen desensitization, that is, a downgrading of the IgE inflammatory response. Treatment may be with, for example, a composition containing purified protein allergens. A mixture of species specific and species non-specific compositions are preferred. Treatment is with a composition containing a library of purified antigens, or with recombinant polypeptides or anti-idiotype antibodies which are immunologically identifiable with the protein allergen by virtue of one or more immunogenic epitopes which are immunologically cross-reactive with those on an *H. pylori* protein allergen. One or more allergens contained within DEAE fractions 59, 64, 66, 68, 72 and 74, the preparation of which is described in **Example 1**, may be particularly suitable. Even more preferable are antigens isolated and purified according to the protocol in Table 2. Embodiments of these antigens are identified in Table 4.

Treatment may also be with, for example, allergoids of protein allergens. Methods of preparing allergoids from antigens are known in the art. Typically, mild formalin or glutaraldehyde treatment of the antigen reduces the allergenicity (IgE formation) without affecting the antigenicity (IgG "blocking" antibody formation).

Treatment also can be effected, for example, with compositions containing at least one structural analog of an epitope of a protein allergen, which binds to the corresponding IgE paratope, or a mixture of naturally occurring antigens and analogs. Structural analogs are

organic molecules that can assume the appropriate charge distribution and hydrophobic/hydrophilic characteristics to allow binding to the paratope in a fashion which mimics the immunologic binding of the epitope.

5        When the goal is alleviation of the allergic reaction by immunotherapy in the form of hyposensitization, the treated individual receives injections of a composition comprised of one or more relevant allergens continuously. Treatment is begun at a dosage low enough to avoid any 10 local or systemic reactions, and frequent injections, usually once or twice a week are administered at increasing dosages until the highest dose the patient can tolerate without excessive local or systemic reactions is reached. This is a maintenance dose, which is then 15 continued at less frequent intervals, usually every 1-6 weeks depending upon the individual's response. However, the actual dosage and treatment regimen depends upon the individual treated, and is determined by the person administering the treatment.

20       Sources of antigens suitable for the practice of the present invention include *Helicobacter*, *Bacteroides* and *Streptococcus*.

#### Vaccines

25       In another embodiment of the invention, the immunoreactive polypeptides (excluding allergens and those antigens that are totally or partially homologous to human self-proteins) or structural analogs of epitopes, are prepared into vaccines. A goal of vaccination is augmentation of the immune response. 30       Vaccines may be prepared from one or more immunogenic polypeptides. If made by recombinant technology, these polypeptides are suitably expressed in a variety of host cells (e.g., bacteria, yeast, insect, or mammalian cells). Alternatively, the antigens may be isolated from 35       microbial preparations or prepared synthetically if the amino acid sequence is known.

Preparation of a "customized" vaccine includes generation of an individualized immunological profile for each person who is a vaccine candidate. Biological samples from each prospective patient are tested for 5 immunological reactivity against a library of antigens specific for the disease. The individual immunological profiles include determinations of antigenicity as well as characterization of the levels of specific isotypes of the patient's immune response. The result is a 10 documented response of the immunoglobulin isotypes (IgG, IgA, IgM and IgE) to each antigen of a library of antigens. Only after the initial screening procedure is conducted are the individual antigens which will make up the "customized" vaccine selected. To provide a multi- 15 antigen "customized" vaccine, antigens from a library are selected on the basis of non-toxicity and combined immunogenicity.

To minimize risk of toxicity, selection of the appropriate antigens to include in each "customized" 20 vaccine requires examination of each individual's immunological profile (described herein) on the basis of isotype. An important aspect is identification of antigens which elicit an IgE response in a particular individual because these antigens would elicit an 25 anaphylactic response if administered in a vaccine. These antigens would not be included in the "customized" vaccine to reduce the possibility of anaphylaxis. Another selection criterion is a preference for including 30 antigens in the "customized" vaccine that demonstrate significant IgA and/or IgG humoral responses. This criterion enhances the effectiveness of the vaccine.

An additional consideration in the formulation of a "customized" vaccine is minimization of induction of an 35 auto-immune response. Using sequence information available for the antigens comprising the library, the sequence is compared with the amino acid sequences of other human proteins via computer-aided protein database comparison. With the increase of sequence information

available from the Human Genome Project there is a corresponding increase in the likelihood that potentially cross-reactive antigens will be identified and not included in a vaccine. In the absence of epitopic sequences or in the absence of sequence information for the antigens comprising the library, cross-reactivity of antigens is used to eliminate an auto-immune response. Antigen cross-reactivity can be determined through a variety of techniques. One such technique involves 5 generation of monoclonal antibodies against all antigens comprising the library by techniques of Kohler and Milstein, well known in the art. The monoclonal antibodies can then be used in an immunohistochemistry assay on tissue samples from various human organs. 10 Antigens that correspond to monoclonal antibodies that bind to the human tissue samples in the immunohistochemistry assay would not be included in a 15 vaccine.

The vaccines of the present invention entail the use 20 of many available immunogens selected from a large antigen library in order to provide wide spectrum antigen coverage. By providing a multi-antigen-vaccine, all immunized subjects can attain a sufficiently strong immune response which is also more likely to be effective 25 long-term. Strong antigens act as adjuvants to assist weak antigens to provoke a stronger response. Also, summation of responses to many antigens makes up for weak individual responses. By selecting a suitable set of antigens, the response is made more focused.

30 A customized vaccine is developed from the antigen library of the present invention and administered to a subject as follows, the examples provided are for *H. pylori*-related diseases, but the invention is not limited to those diseases:

35 (1) Screening each subject to be vaccinated for the presence of serum IgE specific for any of the proteins in the antigen library of the present invention;

(2) Not using as immunogens in the vaccine any IgE-reactive proteins detected in (1) for the respective subject because of high anaphylaxis risk;

5 (3) Of the remaining antigens, not using as antigens those in the library that are completely or partially homologous to human proteins such that there would be cross-reactivity, that is eliminating antigens that could be auto antigens. If an antigen from the protein library derived from a

10 microorganism matches the amino acid sequence of a human protein, it is eliminated; if there is a partial match, an acceptable antigen may be created by removing the homologous sequence e.g. by synthesizing a sequence that is non-homologous.

15 (4) Screening the remaining, desirable immunogenic proteins for serum IgA and IgG reactivity. Among subjects known to be carrying *H. pylori*, preference is given toward including in a vaccine those antigens which are already inducing a significant IgA and/or IgG humoral response. Because an immune response favoring gastro-enteric clearing of the bacteria is desired, those proteins already eliciting an IgA response may be more useful in the vaccination process than those not eliciting such a

20 response.

25 (5) Determining which sets of antigens selected to this point also produce cellular immunity, and combining this information with that obtained in (4) to select the clinically most effective set of antigens for

30 the vaccine;

(6) Preparing the vaccine using the customized set of antigens;

(7) Administering the antigenic proteins in any number of ways known in the art in order to effect

35 vaccination. One such method entails administration of the desirable antigens along with a standard adjuvant. Another method uses a library of altered vaccinia viruses, each virus possessing one of the

5 antigens, for example, found in the *H. pylori* antigen library. After ruling out those vaccinia strains carrying the expressed IgE-reactive proteins, and applying the other criteria for antigen selection presented above, the remainder of the viruses are used in a preferred mixture for immunization without the need to use adjuvants for the immunization process.

10 The preparation of vaccines which contain as active ingredients, immunogenic polypeptides or structural analogs having epitopes is known to one skilled in the art. Such methods are used to prepare the vaccines using antigens of the present invention. Typically, such vaccines are prepared as injectable liquid solutions or 15 suspensions. Solid forms suitable for solution in, or suspension in a liquid prior to injection are also prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes.

20 The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Exemplary of suitable excipients are water, saline, dextrose, glycerol, ethanol, and combinations of these. In addition, if desired, the vaccine may contain minor 25 amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-30 threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminylL-alanine-2-(11-21-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-35 ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be

5                   determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing, for example, an *H. pylori* immunoreactive sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

10                  The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of 15 administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active 20 ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, -sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions 25 take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

25                  The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed 30 with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

35                  Vaccines within the present invention are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The

quantity to be administered, which is generally in the range of about 5 micrograms to about 250 micrograms of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize 5 antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, 10 or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reenforce the immune response, for 15 example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen is also, at least in part, determined by the need of the individual and be dependent upon the judgment of the practitioner.

20 Antibodies to Bacterial Antigens

In another embodiment of the invention, a polypeptide containing one or more epitopes immunologically identifiable with epitopes of a bacterial antigen, for example, an *H. pylori* allergen, are used to prepare 25 antibodies to *H. pylori* epitopes, using the polypeptide as an immunizing agent, and methods known to those of skill in the art. The antibodies prepared include purified polyclonal antibodies, single-chain antibodies, monoclonal antibodies, antibody fragments, and the like. 30 These antibodies are used, for example, for purification by affinity chromatography of polypeptides of interest. More specifically, they are used to purify polypeptides containing epitopes immunologically identifiable with epitopes of *H. pylori* allergens, including the allergens 35 themselves.

In turn, antibodies to bacterial epitopes are used for the preparation of anti-idiotype antibodies. These

anti-idiotype antibodies are comprised of a region which mimics the epitope of the allergen. Anti-idiotype antibodies are synthesized using methods known in the art, and generally use antibodies directed to epitopes as an immunizing agent. In an illustrative embodiment, epitopes are from *H. pylori* as described herein.

Anti-idiotype antibodies are useful in immunotherapy of individuals sensitive to allergens, as well as for the purification of and/or detection of antibodies directed to antigens containing epitopes which immunologically cross-react with the anti-idiotype antibodies.

The immunogenic polypeptides prepared as described above are used to produce polyclonal and monoclonal antibodies. If polyclonal antibodies are desired, a selected mammal (mouse, rabbit, goat, horse, and the like) is immunized with an immunogenic polypeptide bearing an epitope(s). Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to the epitope contains antibodies to other antigens, the polyclonal antibody is purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. See for example, Mayer and Walker (1987). Polyclonal antibodies are isolated from an individual previously infected with the bacterial antibodies and are purified by the methods discussed above.

Monoclonal antibodies directed against specific microbial epitopes are readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See U.S. patents No. 4,341,761, No. 4,399,121, No. 4,427,783, No. 4,444,887, No. 4,466,917, No. 4,472,500, No. 4,491,632 and No. 4,493,890. Panels of monoclonal antibodies

produced against a specific set of epitopes are screened for various properties, that is, for isotype, epitope affinity and the like.

5       Antibodies, both monoclonal and polyclonal, which are directed against microbial epitopes are particularly useful in diagnosis, and those which are neutralizing are useful in passive immunotherapy. Monoclonal antibodies, in particular, are useful to raise anti-idiotype antibodies.

10       Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired. See, for example, Nisonoff (1981), and Dreesman et al. (1985). Techniques for raising anti-idiotype antibodies 15 are known in the art. See, for example, Grych (1985), MacNamara et al. (1984), and Uytdehaag et al. (1985). These anti-idiotype antibodies are also useful for treatment, vaccination and/or diagnosis of *H. pylori* induced gastritis and/or gastroduodenal ulcers, as well 20 as for an elucidation of the immunogenic regions of *H. pylori* antigens.

#### Cloning and Expression of Antigen Proteins

Obtaining bacterial proteins as a source of purified antigens by direct extraction of proteins from a 25 microorganism is not optimal. For many species, including *H. pylori*, it is difficult to grow adequate amounts of the microorganism in culture to provide libraries of purified antigens. A better method to obtain relatively large amounts of purified antigens is to 30 produce them by recombinant genetic methods. However, even recombinant methods of producing antigens by cloning the genes encoding the antigens and expressing the genes in a host, will not always yield maximum 35 quantities of protein. It therefore is preferable to clone the genes encoding these proteins and express them in a host such as *E. coli* in such a way that they can be expressed in high amounts, for example, after induction

with isopropyl B-D-thiogalactopyranoside (IPTG) (Sambrook et al., 1989).

As an illustrative embodiment, the partial amino acid sequences of *H. pylori* antigen proteins that are responsible for development of specific IgE in the patients are identified. To determine an amino acid sequence, electrophoresis of the proteins on polyacrylamide gel is used to separate the proteins from minor impurities. For a specific protein, electrophoretic transfer onto PVDF (polyvinylidene fluoride, Millipore, Bedford, MA) membrane, identification of the protein by staining with Coomassie blue R-250, excision of the protein band, and sequencing on amino acid microsequencer, is a suitable method. If the amino-terminus of the protein is not blocked, microsequencing is suitable. If the amino terminus is blocked, the protein is subjected to cyanogen bromide cleavage which specifically cleaves the protein at the internal methionine resides. This step generates oligopeptides which are separated on a polyacrylamide gel and subjected to amino acid sequencing as described above.

On the basis of partial amino acid sequence information, oligonucleotide primers are designed which are used to clone the genes which encode specific antigen proteins. The Polymerase Chain Reaction (PCR) technique is suitable for this purpose. The isolated genes are cloned into procaryotic expression systems such as Glutathione S-transferase (GST) Gene Fusion system (Pharmacia) or Qiaxpress system (Qiagen Inc.). The GST Fusion system is designed for IPTG inducible, high-level expression of genes as a fusion protein with glutathione S-transferase at the amino-terminus. This fusion protein is purified readily from *E. coli* lysates by affinity chromatography using glutathione-sepharose. The glutathione S-transferase protein at the amino-terminus is selectively cleaved from the desired protein by site-specific protease because the expression plasmids have

the specific recognition sequence for the protease at the junction. The Qiaexpress system allows the production of recombinant protein containing an amino- or carboxy-terminal affinity tag consisting of six adjacent 5 histidine residues (6XHis). The engineered 6XHis tag allows a single-step purification by nickel-chelate affinity chromatography. Some high molecular weight fusion proteins when produced in high amounts tend to aggregate causing insolubility. In such cases the former 10 expression system has a limitation in applying affinity chromatography techniques for purification. In the Qiaexpress system, however, insoluble fusion protein is dissolved with either urea or guanidium hydrochloride and purified on Ni-chelate affinity chromatography.

15 If not otherwise indicated, the practice of the present invention suitably employs conventional techniques of protein purification, microbiology, molecular biology, and immunology, which are within the skill of the art. Such techniques are explained fully in 20 the literature.

The following examples are provided for illustrative purposes only, and not to limit the scope of the present invention. In light of the present disclosure, numerous 25 embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

#### Example 1

##### Isolation of *H. pylori* Protein Allergens and Covalent Coupling of the Allergens to Paper Discs

###### A. Processing of *H. pylori*

30 Four grams, wet weight, of *H. pylori* (ATCC strain 43504; ATCC, Bethesda, MD, USA) were cultured essentially by the method of Smibert (1978). More specifically, *H. pylori* obtained from the American Type Culture Collection, ATCC No. 43504, was removed aseptically from 35 its vial, suspended in 1 ml sterile Difco Brucella broth, and transferred by an in inoculating loop to 3 separate Brucella Agar plates (Anaerobe Systems, San Jose, CA).

The plates were incubated at 35°C for 5 days in a microaerophilic atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% O<sub>2</sub>. After incubation the plates were removed and examined. Tiny grayish-white colonies were observed.

5 Microscopic examination of a Gram-stained smear showed large oxbowshaped and loops of Gram-negative, spiral-shaped organisms, approximately 5 microns long, which are typical of *H. pylori*.

10 *Helicobacter pylori* in colonies from the 5-day plate were transferred to a fresh set of Brucella plates, and the plates were incubated microaerophilically at 35°C for 3 to 5 days. After 3 days a more luxuriant growth of *H. pylori* colonies occurred. These colonies were used as the inoculum for a broth seed culture.

15 A broth seed culture was prepared by transferring to several 10 ml screw-capped tubes 5 ml sterile Brucella broth with 5% horse serum (GIBCO BRL), and colonies collected by swab from the plates. All tubes were incubated at 35°C under a microaerophilic atmosphere for 20 3 to 5 days. If a heavy degree of turbidity was observed in the tubes after this period, the culture was examined for purity by microscopic examination of a Gram stained slide.

25 The broth seed culture was used as an inoculum for one liter of sterile Difco Brucella broth containing 5% horse serum. The inoculated culture was grown in a 3 liter flask by incubation at 35°C in a microaerophilic atmosphere for 3 to 5 days. When a moderate degree of turbidity was observed, the culture was checked for 30 purity as described above. One liter of culture generally yielded an unwashed cell amount of about 2.0 grams.

35 In order to isolate the protein allergens, the living organisms from the liter culture were pelleted by centrifugation at 3,000 RPM, 4°C for 15 minutes. The attenuated bacteria were then repelleted by similar centrifugation. The pellet was resuspended in 20 ml of cold buffer containing 50 mM sodium phosphate, pH 7.3,

150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 micrograms/ml PMSF and 100 micrograms/ml of benzamidine. Ten mL of 150-210 micron, acid-washed glass beads (Sigma, St. Louis, MO, USA) were added, and the resulting suspension then was  
5 sonicated at setting No. 7 by means of a 400 Watt Branson Sonifier II ultrasonic cell disrupter with a regular tip. The suspension thus was sonicated for 15 minutes while being cooled in a methanol ice bath. The resulting mixture was then centrifuged as above and the supernatant  
10 saved.

**B. Gradient Centrifugation**

The supernatant was centrifuged for 1 hour at 100,000 g and 4°C, in a Beckman SW 40Ti rotor (Beckman, Palo Alto, CA, USA). To the resulting supernatant was  
15 added 0.456 cjm/ml of RbCl (Aldrich Chemical Co., Milwaukee, Wis., USA). The solution was then centrifuged at 4°C for 48 hrs. in a Beckman 70 Ti rotor (the first 24 hours at 65,000 RPM and the second 24 hour at 48,000 RPM). The supernatant contents of each gradient tube  
20 were collected in ten equal fractions beginning at the bottom of each tube. The pellet in each tube representing most of the residual complex carbohydrates and nucleic acids containing in the pregradient supernatant was discarded.

**C. Ion Exchange Chromatography**

Each gradient fraction was dialyzed against 20 mM sodium phosphate buffer, pH 7.0, at 4°C using dialysis tubing with a 1,000 MW cutoff. An approximation the protein content per fraction was made by  
30 spectrophotometry at a wavelength of 280 nm. Ninety percent of the detected protein was found in fractions 2 through 6, inclusive; these fractions were pooled. The pooled fractions were then loaded onto a Bio-Sil DEAE analytical anion exchange HPLC column (BioRad, Richmond, CA, USA) and a 30 minute linear gradient run achieving  
35 100 per cent Buffer B at the end of the gradient. The

equilibrating buffer (Buffer A) was 20 mM Sodium phosphate, pH 7.0. The salt containing buffer (Buffer B) was 20 mM sodium phosphate, pH 7.0, with 1.0 M NaCl. The eluted fractions were collected and the protein of each 5 quantified as before. The flow-through (void) fraction containing macromolecules and cationic molecules was loaded onto a Bio-Sil SP cation exchange column (BioRad) and run under the exact gradient conditions as for the DEAE run. The resulting eluted fractions were also 10 quantified for protein.

**D. Covalent Coupling of *H. pylori* Proteins to Paper Discs**

CnBr activated paper discs were made essentially by the method of Ceska (1972). More specifically, paper 15 discs (diameter 6 mm) were cut with a punch from Schleicher and Schuell 589 red ribbon filter paper. The discs were allowed to swell for 30 minutes in water. CNBr solution (5 per cent in water), was added and mixed with a mechanical stirrer for 3 minutes in a water bath at 19°C. NAOH (1 M), was added dropwise to maintain the 20 pH in the range of 10.0 to 10.5. The suspension was immediately poured into about a ten-fold excess of cold NaHCO<sub>3</sub> solution (5 mM, 4°C). After thorough mixing, the solution was decanted. The wash with NaHCO<sub>3</sub> solution 25 was repeated eleven times. The paper discs then were washed twice each with 500 ml of 25%, 50%, and 75% acetone in a graded series, followed by washing four times with 500 ml acetone (reagent grade, 4°C). They were then placed on a filter paper under hood ventilation 30 for 3 hours for drying, packaged with desiccant pouches in plastic bags, and stored at -20°C until use.

A sufficient volume was taken from each of the elution samples collected during the ion exchange runs and diluted with 50 mM sodium carbonate buffer, pH 9.6, 35 to yield a 3 ml solution containing 300 micrograms of protein. To each were added 30 CNBr-activated paper discs, and the mixture then was placed under gentle

agitation for 48 hours at 4°C in order to covalently couple the various proteins to their respective discs. The protein discs were washed and blocked with ethanolamine as described by Ceska, *supra*.

5

Example 2**A Modified RAST Procedure for Detecting IgE Specific to *H. pylori* Allergens**

IgE specific for *H. pylori* allergens prepared according to Example 1 was assayed using a modified RAST procedure. Part of the procedure was essentially as described by Nalebuff et al. (1981). More specifically, an aliquot of 100 microliters of serum was incubated overnight with an appropriate allergen disc and washed three times with 50 mM phosphate buffered saline (PBS), pH 7.3, containing 0.1% Tween 20. This was followed by a second overnight incubation with  $^{125}\text{I}$ -labelled anti-IgE which was specific for the De-2 determinant. After being washed, and prior to being counted, the allergen discs were placed into fresh tubes in a gamma counter for the amount of time previously selected by a time control.

The time control consisted of 25 units of WHO-standardization IgE that was run against a PRIST anti-IgE disc for the time needed for the IgE to bind 25,000 counts. This time was used in the counting of all subsequent tests.

Background levels for individual patients were determined by running each Protein A scrubbed serum against 4 blank discs, and calculating a median value representing the individual's background. Values twice this background level or greater were deemed positive. Determining the individual background level for each patient increases the precision of the assay, since it takes into account the variability corresponding directly to total serum IgE (not just that specific for the bacterial allergens).

As shown in FIG. 1, in order to detect *H. pylori* IgE, it was useful to scrub the serum samples to remove most

IgG and IgA antibodies before incubation with discs containing *H. pylori* protein allergens.

Scrubbing was by incubation with recombinant Protein A/Sepharose (Zymed, S. San Francisco, California USA). More specifically, two ml of serum per one ml of Protein A/Sepharose were incubated with agitation for 1 hr. The suspension was then centrifuged at 1500 RPM for 15 min. and the serum supernatants collected.

The results in FIG. 1 were obtained by taking two aliquots of the same serum from a patient with documented gastritis and *H. pylori* colonization, and subjecting one of these aliquots to the scrubbing procedure. The scrubbed and unscrubbed samples from equivalent amounts of serum were then subjected to the remainder of the RAST procedure using discs containing *H. pylori* protein allergens, as described above. In the figure, the serum IgE levels detected in the scrubbed (open squares) and unscrubbed samples (closed circles) are compared. As seen from the graph, the scrubbed samples allowed the binding of IgE to the *H. pylori* protein allergens which had eluted from the DEAE column with a peak at fraction number 66. This binding was not detected in the unscrubbed sample. A repeated assay yielded similar results.

25

Example 3Analysis of Patient Sera for *H. pylori* Specific IgE

Some aspects of the present invention stem from the discovery using the present invention that individuals with chronic gastritis or gastroduodenal ulcers have serum IgE specific for protein allergens of *H. pylori*, implicating hypersensitivity to this microorganism in the etiology of the diseases.

*H. pylori* is most likely an innocuous colonizer of the gastric mucosa. It dwells just beneath the protective mucous layer and probably feeds from it without much harm to the host or to the host's protective

defenses against the gastric acid. The inflammatory process recognized in chronic gastritis results in those individuals who possess the genetic proclivity toward allergy and then have the necessary MHC II antigen framework for presenting the *H. pylori* allergenic proteins as allergens. A qualitative and/or quantitative reduction in the secretion of protective mucus by the goblet cells probably occurs thus making the underlying mucosa vulnerable. In addition, a likely increase in local histamine production may take place in response to the allergic reaction. The histamine is absorbed into the vascular plexus of the stomach thus leading to an increase in gastric acid production. These two phenomena may together result in increased irritation of the early gastric lesions and, along with the constant allergic reaction to *H. pylori*, lead to lesion enlargement and chronicity.

Immunoassays were designed to detect an *H. pylori* induced allergic reaction in individuals. In one aspect, these immunoassays utilize purified protein subfractions (allergens), and are preferable to endoscopy because they may be performed *in vitro* and are relatively non-invasive. In addition, the discovery allows for a novel treatment of these diseases; that is, immunotherapy with compositions comprised of at least two purified protein allergens of *H. pylori*, and/or with an allergoid of a protein allergen of *H. pylori*.

Ten consecutive gastritis/GI ulcer patients that were disease positive by endoscopy, two patients without lesions by endoscopy, and twelve apparently asymptomatic control patients were tested using the modified RAST procedure with scrubbing, as described in **Example 2** and antigens prepared in accordance with **Example 1**.

All ten disease positive patients had measurable quantities of *H. pylori* specific IgE in their sera. The two normal endoscopy patients were IgE negative, and six of twelve asymptomatic control subjects were also IgE positive to some of the HPLC eluted proteins. As shown

in FIG. 2, each IgE positive patient appeared to react differently to the various HPLC fractionated proteins.

5 The prevalence of IgE positive reactivity toward the individual chromatographed fractions for each positive patient in the "asymptomatic" and "gastritis" patients was examined. There were several *H. pylori* protein fractions to which the disease group patients reacted with greater exclusivity than the "asymptomatic" patients. This more exclusive reactivity was with DEAE 10 fractions 59, 64, 66, 68, 72 and 74.

15 FIG. 3 shows a plot of the net total IgE immunological reactivity of serum from control and gastritis patients using all available *H. pylori* protein fractions isolated from an HPLC DEAE column. FIG. 4 is a plot of the net total IgE immunological reactivity of serum from control and gastritis patients with the proteins in fractions 59, 62, 65, 70, 64, 68, 71, 73, and 74.

#### Example 4

##### Quantifying Specific IgE in Nasal Polyposis

20 Patients with chronic paranasal sinus disease exhibit a high positive prevalence of bacteria-specific serum IgE. Quantitation of IgE was used to discriminate among classes of patients.

25 A modified radioallergosorbent test method was employed wherein each serum sample was absorbed with recProtein A to remove competing non-IgE antibodies, and purified proteins extracted from 16 individual bacterial genus were used as potential allergens.

30 Twenty-four patients with nasal polyposis and 14 with chronic sinusitis, all refractory to conventional medical therapy and requiring endoscopic sinusotomies, were tested. Tested as controls were 10 subjects with chronic allergic rhinitis, without a history of chronic sinus 35 disease, and possessing total serum IgE and inhalant-specific IgE levels equal to or higher than the patient group.

The results indicated that:

(1) Pretreatment of serum samples with recProtein A resulted in an increase of bacteria-specific radioallergosorbent test sensitivity.

5 (2) Seventeen of 24 patients with polyps, eight of 14 with chronic sinusitis, and one of 10 with chronic allergic rhinitis were determined to be IgE positive when tested with this assay.

From these results, it was concluded that:

10 (1) Bacteria-specific serum IgE can be quantified;

(2) Most patients with nasal polyposis and/or chronic sinusitis possess bacteria-specific IgE in their serum, while subjects with only allergic rhinitis do not; and

15 (3) Multiple bacterial species isolated from chronically infected sinuses are capable of inducing IgE-mediated sensitization.

Example 5

Immunoglobulin Absorption Experiment

20 Autoradiographic western blots were performed in which IgE-H. pylori protein reactivity was measured employing serum from four individual peptic ulcer disease patients and six different IgE-reactive H. pylori antigens (Ag1 through Ag6). In each case, individual 25 patient sera were: (1) tested neat; (2) tested after absorption of all serum IgA from the serum sample using immobilized Jaclin lectin (Product # 20395, Pierce Co.) see Kumar et al., 1982; Roque-Barriera and Campos-Neta, 1985; Mesticky et al., 1971; and Van Kamp, 1979; (3) 30 tested after absorption of all IgG from the serum sample using immobilized recombinant protein G; and (4) tested after absorption of both IgA and IgG from the serum sample. The net IgE signal for each serum condition was determined in radioactive counts per minute.

Table 5: IgE *H. pylori* Protein Reactivity in Serum from Patients with Peptic Ulcers

		Antigens					
		<u>Aq1</u>	<u>Aq2</u>	<u>Aq3</u>	<u>Aq4</u>	<u>Aq5</u>	<u>Aq6</u>
5	<u>Patient 1:</u>						
	neat serum	81	0	0	0	0	0
	minus IgA	97	0	0	0	0	0
	minus IgG	102	0	0	0	0	0
	minus IgA+IgG	74	0	0	0	0	0
10	<u>Patient 2:</u>						
	neat serum	0	0	0	0	110	0
	minus IgA	0	0	17	0	121	0
	minus IgG	0	0	58	0	160	0
	minus IgA+IgG	0	0	34	0	143	0
15	<u>Patient 3:</u>						
	neat serum	0	54	390	116	1384	104
	minus IgA	0	85	404	120	1525	146
	minus IgG	0	182	526	157	1875	148
	minus IgA+IgG	0	103	407	136	1203	79
20	<u>Patient 4:</u>						
	neat serum	0	0	102	0	800	0
	minus IgA	28	0	130	17	882	0
	minus IgG	0	0	212	0	1091	0
	minus IgA+IgG	85	0	137	60	698	0
25	As can be seen from the results in Table 5, the highest reactivity in most of serum tested was after "scrubbing" with immobilized recombinant protein G (i.e. removing IgG).						

MATERIALS AND METHODS

## Methods for Purification of Bacterial

## Antigens into Subfractions:

## Bacterial Protein Extraction with SDS,

Precipitation with Acetone

5

## I. Extraction with 0.1, 1.0 and 10.0 % SDS (Sodium Dodecyl Sulfate) Solutions

A. 0.1% SDS Extraction:

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- 1) For each mL of PBS washed bacterial pellet add 5 mL of 0.1% SDS/20 mM Tris-HCl, pH 7.0 containing 1 mM each of EDTA(ethylenediaminetetraacetic acid), EGTA (ethyleneglycol-bis-tetraacetic acid), leupeptin, Benzamidine and PMSF (phenylmethylsulfonyl fluoride).
- 2) Stir contents at moderate speed for 15 min.
- 3) Centrifuge at 30,000 RPM for 60 min, 4°C.
- 4) Aliquot and freeze supernatant until needed.

15

B. 1.0% SDS Extraction:

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- 1) To remaining precipitate add, for each mL of pellet, 6 mL of 1.0% SDS/20 mM Tris-HCl containing 1 mM each of EDTA, EGTA, leupeptin, benzamidine and PMSF.
- 2) Stir at moderate speed for 15 min. (Solution should be quite viscous.)
- 3) Centrifuge at 30,000 RPM for 60 min, 4°C.
- 4) Aliquot viscous supernatant and set aside on ice.
- 5) To the pellet (and slight amount of remaining viscous supernatant) add another 2 mL of 1.0% SDS extraction buffer per mL of original pellet.
- 6) Stir briefly.
- 7) Centrifuge at 30,000 RPM for 30 min, 4°C.
- 8) Pool viscous supernatants.

9) Slowly add 1.0M Glycine buffer, pH 2.2, to viscous supernatant while stirring until solution has lost its viscosity ( should occur as solution reaches pH 2.2).

5 10) Centrifuge processed supernatant at 30,000 RPM for 60 min, 4° C. Discard any precipitate.

11) Aliquot supernatant and neutralize with 1.0 M NaOH while stirring.

10 12) Centrifuge resulting suspension at 30,000 RPM for 60 min, 4° C.

13) Aliquot and freeze supernatant until needed. Discard precipitate.

C. 10.0% SDS Extraction:

15 1) To each mL of pellet from steps B-7 add 6 mL of 10% SDS/20 mM Tris-HCl containing 1 mM each of EDTA, EGTA, leupeptin, benzamidine and PMSF.

20 2) Stir at moderate speed for 15 min. (Solution should be quite viscous.)

3) Centrifuge at 30,000 RPM for 60 min, 4° C.

4) Aliquot viscous supernatant and set aside on ice.

25 5) To the pellet (and slight amount of remaining viscous supernatant) add another 2 mL of 1.0% SDS extraction buffer per mL of original pellet.

6) Stir briefly.

7) Centrifuge at 30,000 RPM for 60 min, 4° C.

30 8) Pool viscous supernatants. Discard precipitate.

9) Slowly add 1.0M Glycine buffer, pH 2.2, to viscous supernatant while stirring until solution has lost its viscosity ( should occur as solution reaches pH 2.2).

35 10) Centrifuge processed supernatant at 30,000 RPM for 60 min, 4° C. Discard any precipitate.

- 11) Aliquot supernatant and neutralize with 1.0 M NaOH while stirring.
- 12) Centrifuge resulting suspension at 30,000 RPM for 60 min, 4° C.
- 5 13) Aliquot and freeze supernatant until needed. Discard precipitate.

**II. Acetone Precipitation of Proteins in 0.1, 1.0 & 10.0% SDS Extracts:**

**A. 0.1% SDS Extract:**

- 10 1) 50% Acetone-precipitated Proteins
  - a. To each mL of protein extracted from step I-A-4 slowly add (while stirring) 1.0 mL of room temperature HPLC grade acetone.
  - 15 b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4° C.
  - c. Aliquot supernatant and set aside for next precipitation step.
  - d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0.
  - 20 e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).
  - f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.
  - 25 g. Freeze until needed.
- 2) 85.0% Acetone-precipitated Proteins
  - a. To each mL of supernatant from 50% precipitation step slowly add (while stirring) 4.67 mL of room temperature, HPLC grade acetone.
  - 30 b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4°C.
  - c. Aliquot and discard supernatant.

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d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0 and gently agitate to resolubilize precipitate.

e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).

f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.

g. Freeze until needed.

B. 1.0% Extract

1) 30.0% Acetone-precipitated Proteins

a. To each mL of protein extract from step I-B-11 slowly add (while stirring) 0.429 mL of room temperature, HPLC grade acetone.

b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4° C.

c. Aliquot supernatant and set aside for next precipitation step.

d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0.

e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).

f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.

g. Freeze until needed.

2) 35.0% Acetone-precipitated Proteins

a. To each mL of supernatant from 30% precipitation step slowly add (while stirring) 0.109 mL of room temperature, HPLC grade acetone.

b. Centrifuge resulting mixture at 4,000 RPM for 30 minutes, 4°C.

c. Aliquot and discard supernatant.

5

d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0 and gently agitate to resolubilize precipitate.

e. Place resulting SDS solution in boiling water bath for 5 minutes (to inactivate any proteases).

f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.

10 g. Freeze until needed.

15 3) 53.0% Acetone-precipitated Proteins

a. To each mL of supernatant from the 35% precipitation step slowly add (while stirring) 0.59 mL of room temperature, HPLC grade acetone.

b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4° C.

c. Aliquot and discard supernatant.

20 d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0 and gently agitate to resolubilize precipitate.

e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).

f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.

25 g. Freeze until needed.

30 4) 85.0% Acetone-precipitated Proteins

a. To each mL supernatant from 53% precipitation step slowly add (while stirring) 4.542 mL of room temperature, HPLC grade acetone.

b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4° C.

c. Aliquot and discard supernatant.

35 d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0

and gently agitate to resolubilize precipitate.

- e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).
- f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.
- g. Freeze until needed.

C. 10.0% Extract:

10 1) 39.0% Acetone-precipitated Proteins

15 a. To each mL of protein extract from step I-C-4 slowly add (while stirring) 0.64 mL of room temperature, HPLC grade acetone.

20 b. Centrifuge resulting mixture at 4,000 RPM for 30 minutes, 4°C.

25 c. Aliquot supernatant and set aside for next precipitation step.

30 d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0.

35 e. Place resulting SDS solution in boiling water bath for 5 minutes (to inactivate any proteases).

f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.

g. Freeze until needed.

2) 43.0% Acetone-precipitated Proteins

30 a. To each mL supernatant from 39% precipitation step slowly add (while stirring) 0.115 mL of room temperature, HPLC grade acetone.

35 b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4°C.

c. Aliquot and discard supernatant.

d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0

and gently agitate to resolubilize precipitate.

- 5 e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).
- f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.
- g. Freeze until needed.

10 3) 53.0% Acetone-precipitated Proteins

- 15 a. To each mL of supernatant from the 35% precipitation step slowly add (while stirring) 0.374 mL of room temperature, HPLC grade acetone.
- b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4°C.
- c. Aliquot and discard supernatant.
- d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0 and gently agitate to resolubilize precipitate.

- 20 e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).

- f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.
- g. Freeze until needed.

25 4) 85.0% Acetone-precipitated Proteins

- 30 a. To each mL of supernatant from 53% precipitation step slowly add (while stirring) 4.539 mL of room temperature, HPLC grade acetone.
- b. Centrifuge resulting mixture at 4,000 RPM for 30 min, 4°C.
- c. Aliquot and discard supernatant.
- d. To each cc of precipitate, add 5.0 mL of 0.1% SDS in 20 mM Tris-HCL, pH 7.0 and gently agitate to resolubilize precipitate.

5

- e. Place resulting SDS solution in boiling water bath for 5 min. (to inactivate any proteases).
- f. When cooled, dialyze solution against 1mM Tris HCL using 3,500 MW tubing.
- g. Freeze until needed.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS Page)**

1. **Stock Solutions**

10 a) **Acrylamide solution (30:0.8)**  
Acrylamide 30.00 g  
Bisacrylamide 0.80 g  
Dissolve in deionized water and make volume to 100 ml. Store in brown or aluminum foil wrapped bottle at 4°C.

15 b) **Tris-HCL buffer, 1.0 M, pH 8.8**  
Dissolve 12.1 g of Tris in 70 ml of deionized water, adjust pH to 8.8 with 1N HCL and make volume to 100 ml. Store at 4°C.

20 c) **Tris-HCL buffer, 1.0 M, pH 6.8**  
Dissolve 12.1 g of Tris in 60 ml of deionized water, adjust pH to 6.8 with 1N HC1 and make volume to 100 ml. Store at 4°C.

d) **10% sodium dodecyl sulfate (SDS)**  
Dissolve 10 g lauryl sulfate (SDS) in 100 ml of deionized water. Store at room temp.

25 e) **10% Ammonium per sulfate (APS)**  
Dissolve 500 mg of ammonium per sulfate in 5ml of deionized water. Store frozen at -20°C.

30 f) **Tetramethylethylenediamine (TEMED)**:  
Available commercially

g) **Water saturated n-butanol**  
Add 10 ml of deionized water into 50 ml of n-butanol, had shake and let it stand till two

phases are separated. Use n-butanol phase (top one).

h) 2X SDS sample buffer

	Tris-HCL, 1.0 M, pH 6.8	6.24 ml
5	SDS, 10%	10.00 ml
	Glycerol	10.00 ml
	Bromophenol blue	0.25 g
	Make up volume to 50 ml with deionized water.	
	Store at room temp. Just before use, add 50 $\mu$ l	
10	of 2-mercaptoethanol to 1.0 ml of above buffer and mix.	

i) Tank Buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3)

	Tris	12.1 g
15	Glycine	57.6 g
	SDS, 10%	40.0 ml
	Dissolve in deionized water and make volume to 4.0 L.	

j) Staining Solution

20	(0.025% Coomassie Blue R-250, 40% methanol, 1% acetic acid)	
	Coomassie Blue R-250	2.0 g
	Methanol	800.0 ml

Stir until dissolved.

25	Acetic Acid	20.0 ml
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k) Destaining Solution

	(40% methanol, 1.0% acetic acid)	
	Methanol	800.0 ml
	Acetic acid	20.0 ml
30	Make volume to 2.0 L with distilled water.	

2. Assembling Gel Caster

Follow instruction manual for Hoffer SE 600 Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA).

35 3. Preparation of Separation Gel (10.0% acrylamide)

In a 50 ml flask take following:

Tris-HCL, 1.0 M, pH 8.8	9.40 ml
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Acrylamide soln (30:0.8)	8.30 ml
--------------------------	---------

SDS, 10%	0.25 ml
APS, 10%	70.00 $\mu$ l
TEMED	40.00 $\mu$
Water, deionized	6.92 ml

5 Mix and pour between the glass and the alumina plate with the help of pipet. Keep top 2.5 cm empty. Layer about 200  $\mu$ l of water saturated n-butanol and let the gel polymerize for at least 1 hr. Pour off n-butanol from the top of the gel and flush with deionized water. Let drain. Pour stacking gel.

#### 4. Preparation of Stacking Gel

In a 25 ml flask take following

Tris-HCL, 1.0 M, pH 6.8 1.6 ml

15 Acrylamide soln (30:0.8) 2.1  
ml

SDS, 10% 125.0  $\mu$ l

APS, 10% 40.0  $\mu$ l

TEMED 20.0  $\mu$ l

20 Deionized water 8.6 ml

Mix and pour. Insert 15 well comb. Make sure that there are no air bubbles below the comb teeth. Let the gel polymerize for at least 30 min. Remove the comb. Gel is ready for electrophoresis.

## 25 5. Sample Preparation

When protein sample is in solution form: add equal volume of 2X SDS sample buffer (Stock soln (h))  
When protein sample is in dried form: dilute 2X SDS sample buffer to 1X with deionized water and dissolve the dried protein.

Heat in the boiling water bath for 3-5 min, cool at room temp. and then load.

## 6. Sample Loading

Quantity of the sample to be loaded depends on the thickness of the gel and number of the wells. When 1.0 mm thick spacer and 15 well comb are used, one can load up to 80  $\mu$ l of sample.

7. Running the Gel

Run the gel at constant current, 20 mA / gel till Bromophenol blue reached bottom of gel. Remove the gel and prepare to transfer contents to 5 nitrocellulose/other blotting paper or stain.

8. Staining and Destaining

Stain the gel in Coomassie blue R-250 (solution 10 (j) for at least 2-3 hrs. (Overnight staining usually gives better results.) Destain with solution (k) till background of the gel becomes clear. (For best results, one usually changes the destain 2-3 times.)

Western Blot Method

1. SDS-PAGE

15 (a) Gel preparation

10.0 % Acrylamide gel is made according to standard SDS-PAGE protocol. 15 well comb is used. Gels are cast at least 1.0 h before use. Separation gel can be cast on previous day; in such a case, layer 20 water-saturated n-butanol on top of the gel. The stacking gel must be cast on the day gel is to be used.

(b) Sample preparation

Before loading individual acetone-precipitated 25 bacterial protein fractions, determine overall protein concentration of each. Dilute individual fractions with enough SDS sample buffer to attain 25-40 µg of protein per 10 µl of sample to be loaded into each corresponding well of gel.

(c) Electrophoresis

30 Run at 20 mA per gel (constant current) for 4 hr or until bromophenol blue dye just runs out. Remove the gel and process for transfer onto nitrocellulose paper as follows.

2. TRANSFER OF PROTEINS ONTO NITROCELLULOSE PAPER(a) Transfer buffer preparation

5 Dilute 10 X Tris-glycine running buffer to 1x concentration, add 10 % methanol while diluting. Prepare 6.0 L of transfer buffer. 10 X Tris-glycine buffer recipe is given in the SDS-PAGE protocol.

(b) Getting ready for transfer

10 Cut 15X16 cm size nitrocellulose paper. Use gloves while handling nitrocellulose paper. Cut whitman paper # 3 of 16X16.5 cm size, each gel requires six pieces. Pour 1.0 L of transfer buffer in the glass tray and soak foam sponges in the transfer buffer. Make sure that no air bubbles are left in the sponges. Two gel sponges are required for each gel.

15 (c) Gel Equilibration

Remove the gel after electrophoresis is run and mark the right side of gel bottom by cutting corresponding corner. Equilibrate gel in transfer buffer for 10 min. by shaking at low speed.

20 (d) Transfer Assembly

25 Following operations take place in the transfer buffer in the glass tray where sponges are soaking: Open cassette in buffer and keep black grid face up. On top of white grid, place one piece of soaked sponge, two sheets of soaked # 3 Whitman paper, one sheet of soaked nitrocellulose paper, equilibrated gel ( marked side should go to right bottom ), two sheets of soaked # 3 Whitman paper, and soaked sponge. Snap the black grid into white grid. While assembling for transfer make sure that air bubbles are not trapped between nitrocellulose paper and the gel. Transfer this assembly into the transfer chamber filled with transfer buffer. Under cooling, apply 100 volts (constant voltage) for 12.0 hrs. Then apply 1000 volts for an additional 2.0 hrs.

30 35 (e) Blocking the Protein-transferred Nitrocellulose Paper

Stir 5 g of non-fat dry milk in 100 mL of 1X PBS/0.05% Tween20 for about 1 hr and then filter through # 4 coffee filter. Add 0.05 % sodium azide and stir.

Remove the nitrocellulose paper from the transfer assembly and incubate with the filtered 5 % non-fat dry milk, shaking at room temperature for 2.0 hrs.

3. INCUBATION WITH PRIMARY AND SECONDARY ANTIBODY

5 (a) Primary Antibody Preparation and Incubation  
Dilute 200  $\mu$ l of patient or control serum with 1.8 mL of 5 % non-fat dry milk (NFDM) in PBS/0.4 % Tween. Incubate at 4°C, shaking slowly for 20 hrs.

10 Remove blocking solution from protein transferred strips and add the diluted serum samples to each individual strip. Incubate with gentle agitation at room temperature for 20 hrs.

15 Wash the strips five times using 4 mL of PBS/0.1 % Tween20 for each strip each time. Between each washing step, incubate strip with wash buffer with gentle agitation at room temperature for 10 minutes.

After last wash, aspirate and add radio-labelled secondary antibody.

20 (b) Secondary Antibody Incubation  
Dilute  $^{125}$ I-labeled goat anti-human IgE with 5 % non-fat dry milk in PBS/0.2 % Tween20 so as to attain 60,000 CPM/ 50  $\mu$ l diluent solution.

25 Add 2.0 ml of labeled secondary antibody solution to each strip (2.4X10<sup>6</sup> CPM/strip). Incubate under gentle agitation at room temperature for 20 hrs.

Wash strips 6 times each as described in 3-a.

Dry the strips at room temp.

30 Use Fuji BAS 2000 imaging system (Fuji Medical Systems, Stamford, CT) to analyze each strip. Determine individual qualitative and quantitative protein band IgE-reactivity

A Modified RAST Test

Generally, in the RAST test an allergen extract is coupled to cellulose particles or paper discs. A patient's serum containing IgE antibody or a control serum is reacted with the allergen-coupled immunosorbent. After thorough washing, labeled antibody is reacted with

the immunosorbent. After further washing, the label on the separated sorbent is determined and is a measure of the amount of specific serum IgE antibodies to that allergen.

5        In an embodiment, the RAST test is modified to increase its sensitivity by removing IgG and/or IgA antibodies which may interfere with IgE binding to the allergen. This is particularly helpful when measuring serum IgE specific to *H. pylori* allergens that are not purified according to the SDS-acetone method of the 10 present invention. Reactants capable of removing IgG, IgM and/or IgA are known in the art, and include, for example, Protein G, anti-human IgG and anti-human IgA, as well as Protein A. For convenience, these reactants are 15 affixed to a solid substrate, including, for example, Sepharose. The amount of the reactants used is sufficient to remove interfering IgG and IgA, but not the IgE which is to be detected. The determination of the desired amount is by methods known to those of skill in 20 the art.

A method of removing interfering IgG and/or IgA antibodies by incubation of the serum with Protein A is discussed in the Examples, infra. Generally, the amount of Protein A which is used is sufficient to prevent the 25 blocking antibodies from competing with the IgE having the same specificity.

The modified RAST test also includes the use of purified protein allergens. Methods of purifying proteins are known in the art and include, for example, 30 differential extraction, salt fractionation, chromatography on ion exchange resins, affinity chromatography and centrifugation. See, for example, Cooper (1977) and Hancock (1984). If antigens are purified by the SDS-acetone method of the present 35 invention "scrubbing" is not needed.

CITED DOCUMENTS

5 The contents of the documents listed below are incorporated herein by reference, respectively, to the extent that they supplement, explain, provide a background for or teach methodology, techniques or compositions described above.

Ceska, et al. (1972) *J. Allergy and Clin. Immunol.* 49:1.

10 Cooper, T.G. (1977) *Tools of Biochemistry - Methods in Enzymology*. J Wiley & Sons, N.Y.

Czinn, S.J. and Nedrud, J.G. (1991) *Infection and Immunity* 59:2359-2363.

Dreesman et al. (1985) *J. Infect. Disease* 151:761.

15 Eaton, et al. (1989) *Infect. and Immun.* (U.S.) 57:1119

Eaton, K.A. and Krakowka, S. (1992) *Gastroenterology* 103:1580-1586

Evans, D.J. (1989) *Gastroenterology* 96:1004

Graham, D.Y. et al. (1988) *J. Infect. Dis.* 157:777.

20 Grych (1985) *Nature* 316:74.

Hancock, W.S. (1984) *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*. CRC Press, Boca Raton, FL.

25 Hupertz, et al. (1988) *Eur. J. Clin. Microbiol. Infect. Dis.* 7:576

Kumar, G.S., Appukuttan, P.S. and Basu, D. (1982) *J. Biosci.* 4:257-261.

MacNamara et al. (1984) *Science* 226:1325.

Marshall (1983) *Lancet* i:1273.

Mayer and Walker (1987) *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London).

5 Megraud, F. (1993) Epidemiology of *Helicobacter pylori* infection, in *Gastroenterology Clinics of North America* 22:73-88.

Mestecky, J., Julhavy, R. and Kraus, F.W. (1971) *J. Immunol.* 107:605.

10 Nalebuff, et al. (1979) *Otolaryngol. Head Neck Surg.* 87:351.

Nalebuff et al. (1981) *Otolaryngol. Head Neck Surg.* 89:271.

15 Nisonoff, A., et al. (1981) *Clin. Immunol. Immunopathol.* 21:397.

Parsonnet, J. et al. (1991) *New England J. Med.* 325:1127

Peterson (1991) *New England J. Med.* 374:1043.

20 Roque-Barriera, M.C. and Campos-Neto, A. (1985) *J. Immunol.* 134: 1740-1743.

Sambrook, J., Fritsch, EF, Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd ed. Vols. 1,2,3. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

25 Slomiany et al. (1989) *Am J. Gastroenterol* 84:1273.

- 72 -

Smibert (1978) *Ann. Rev. Microbiol.* 32:673.

U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783;  
4,444,887; 4,466,917; 4,472,500; 4,491,632; and  
4,493,890.

5 Van Kamp, G. J. (1979), *J. Immunol. Meth.* 27: 301.

Varga, L. et al. (1992) *Orvosi Hetilap* 133:359-61.

Uytdehaag et al. (1985) *J. Immunol.* 134:1225.

WHAT IS CLAIMED IS:

1. A customized vaccine for a disease related to microbial antigens, said vaccine comprising at least two antigens selected from a library of antigens specific for the disease, wherein said antigens are not allergens that complex with IgE present in a subject to whom the vaccine is to be administered.  
5
2. The vaccine of claim 1, further defined as not including an antigen that is sufficiently homologous to any human protein to raise an antibody that recognizes said human protein.  
10
3. The vaccine of claim 1, wherein the antigens provoke a clinically effective response in the subject to whom the vaccine is administered.  
15
4. The vaccine of claim 1, wherein the microbial antigens are *Helicobacter pylori* antigens.  
15
5. A method of making a vaccine against a disease associated with microbial antigens, comprising the steps of:  
20
  - (a) selecting by the following criteria an antigen to include in the vaccine from an antigen library specific for the disease:
    - (i) serum IgE specific for the antigen is not present in the serum of an individual to be vaccinated; and  
25
    - (ii) the antigen is not sufficiently homologous to a human protein to raise an antibody that recognizes said human protein;
- 30
  - (iii) selecting an antigen not eliminated in (i) or (ii) that elicits an immune

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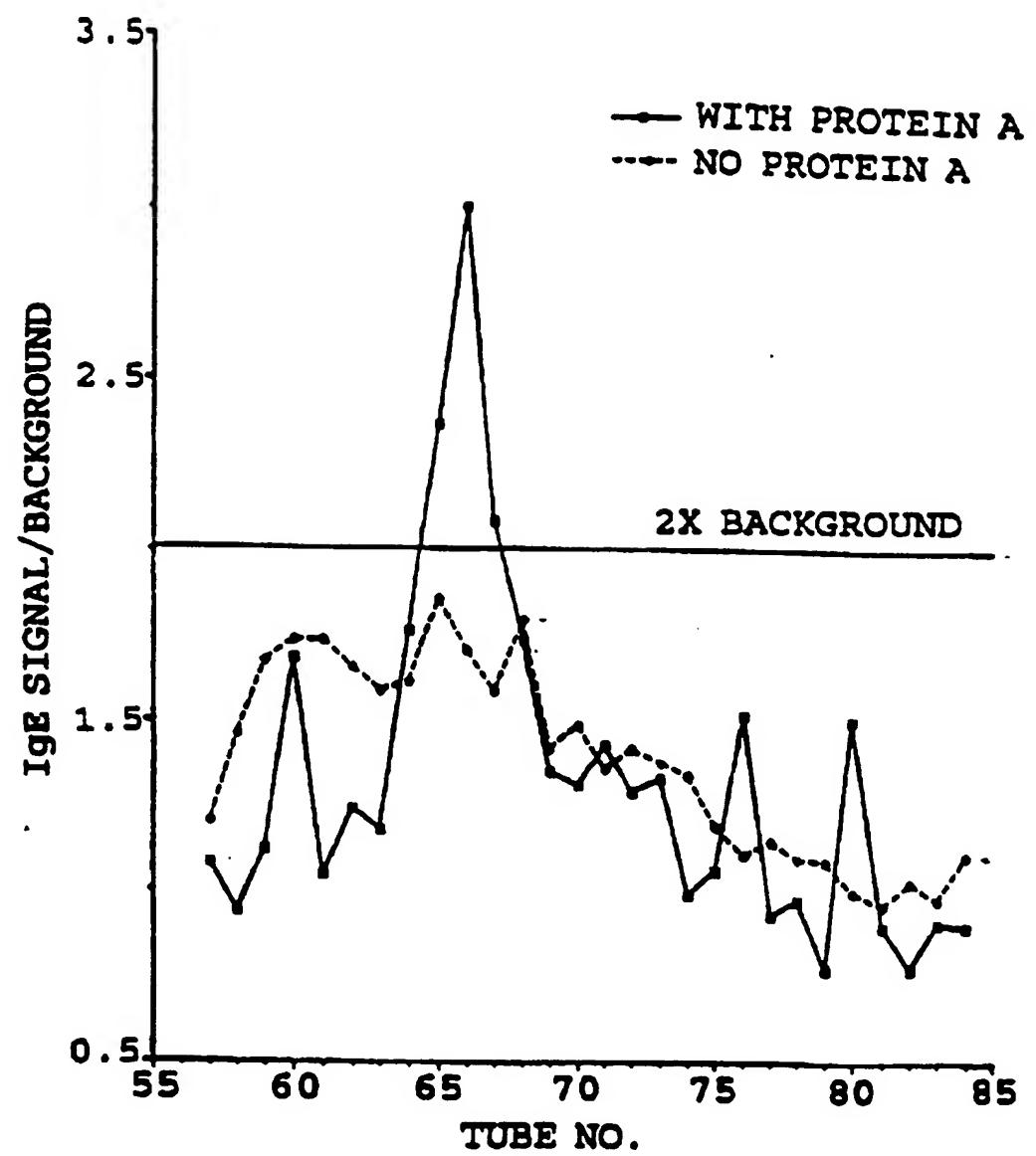
response that is clinically effective; and

(b) using antigens from the antigen library remaining after step (a) to prepare a  
5 vaccine.

6. The method of claim 5, wherein IgG and IgA response to an antigen is used to determine whether an antigen is clinically effective.

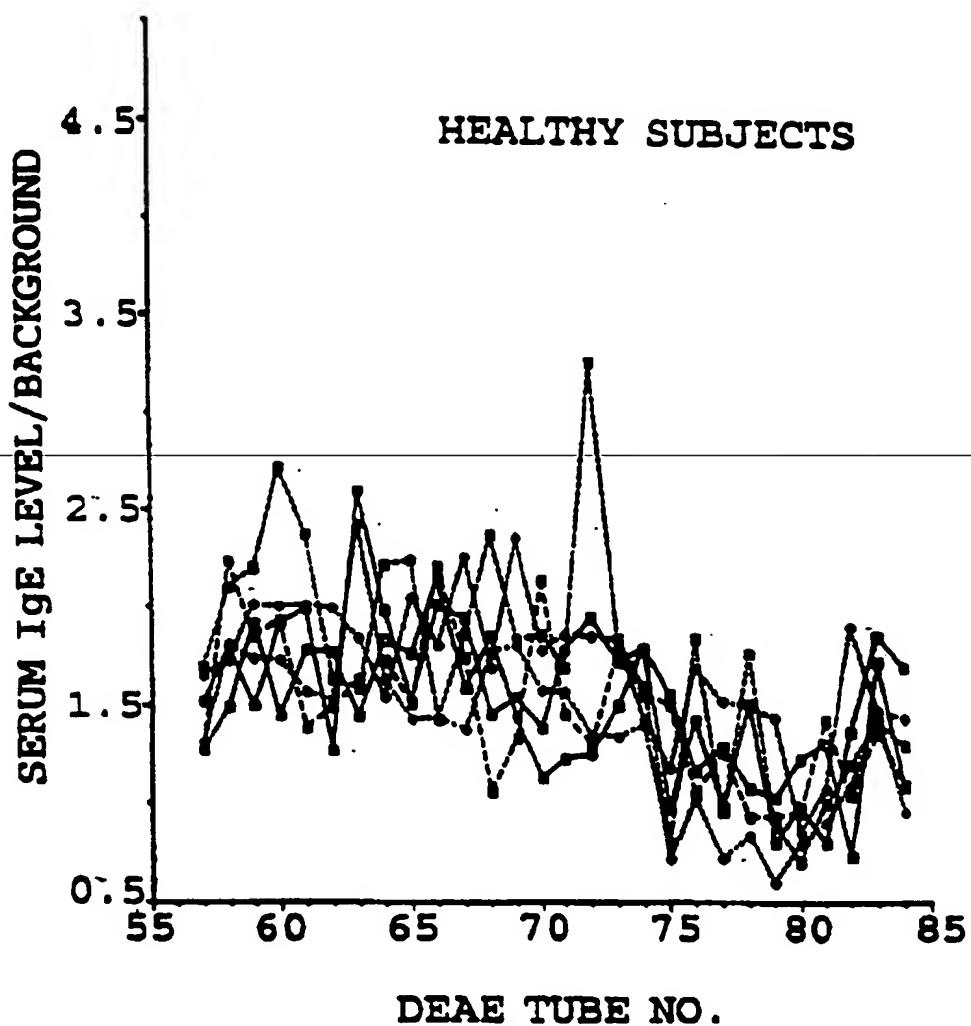
1 / 5

FIGURE 1



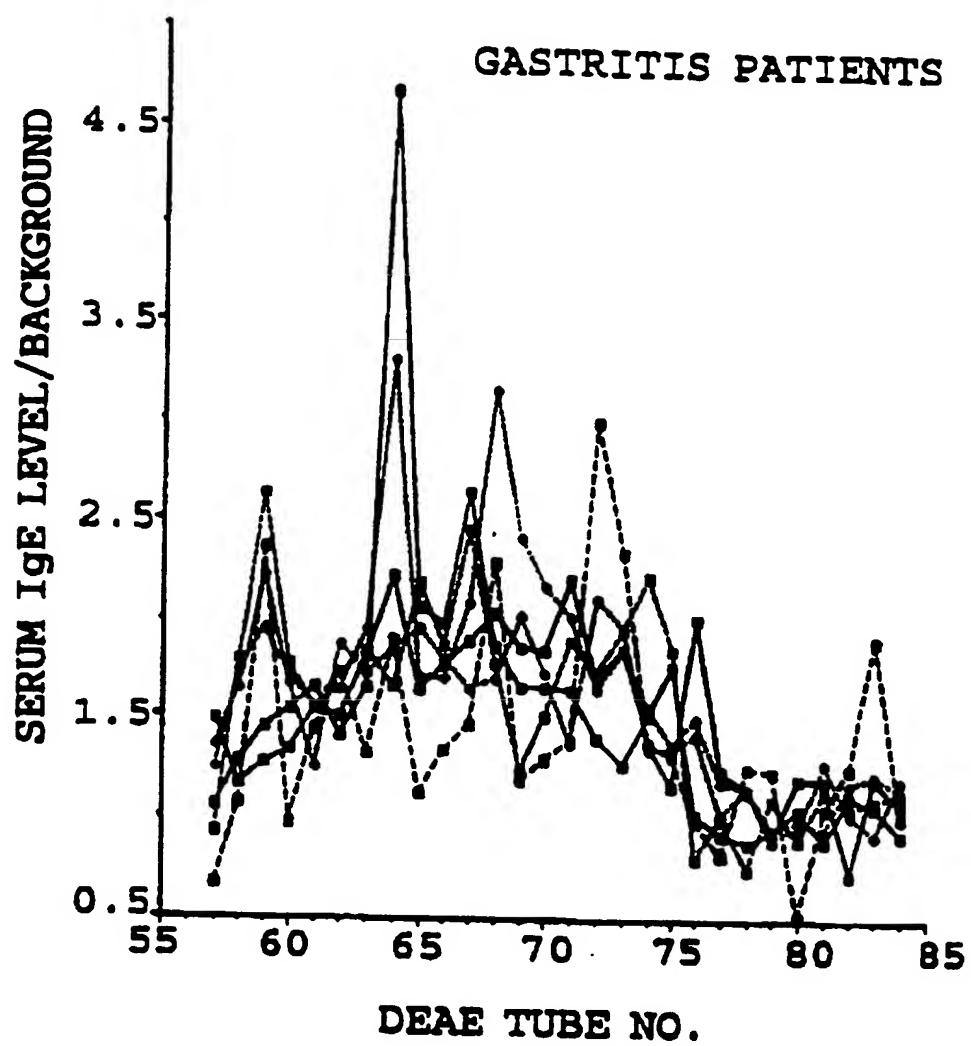
2 / 5

FIGURE 2A



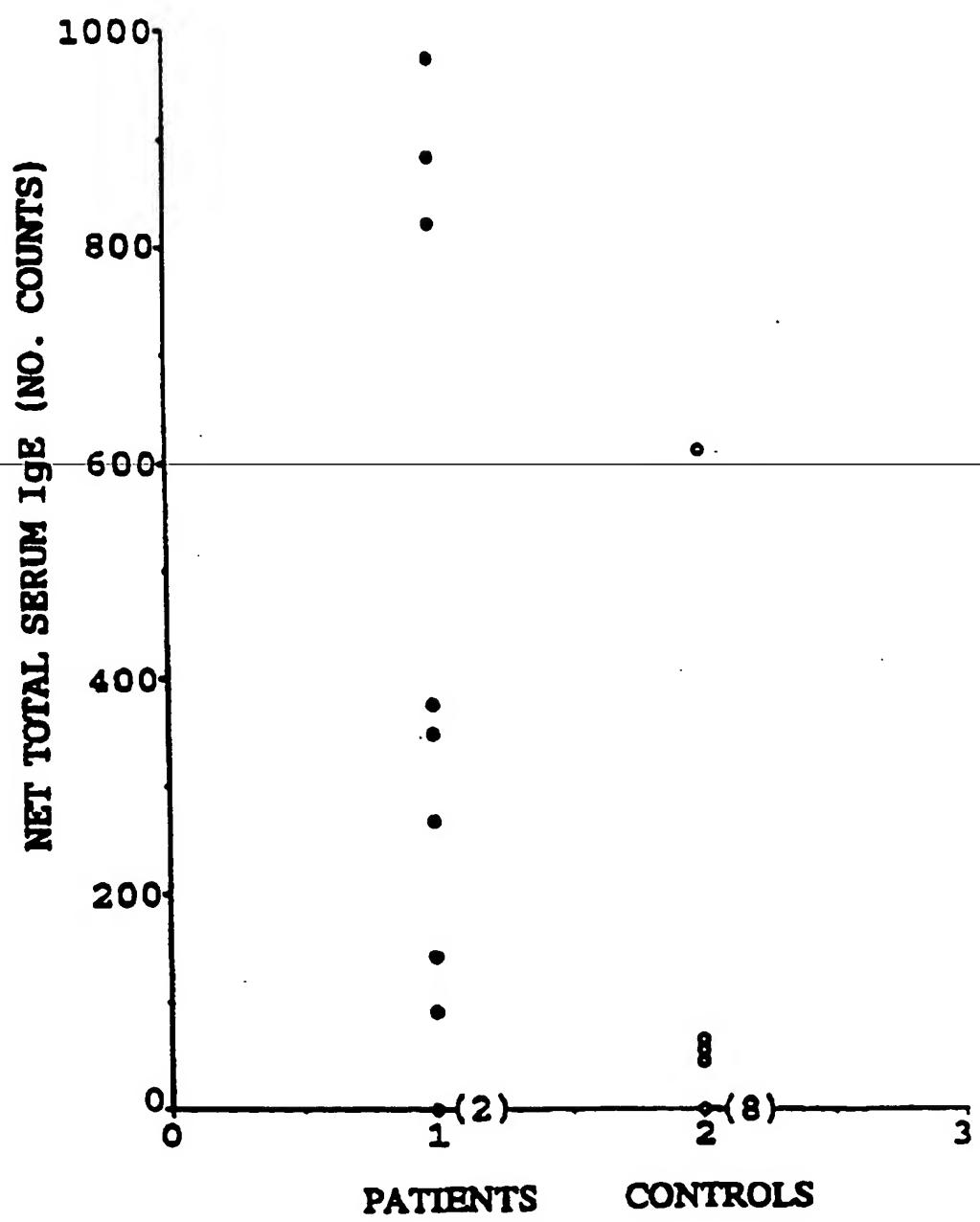
3 / 5

FIGURE 2B



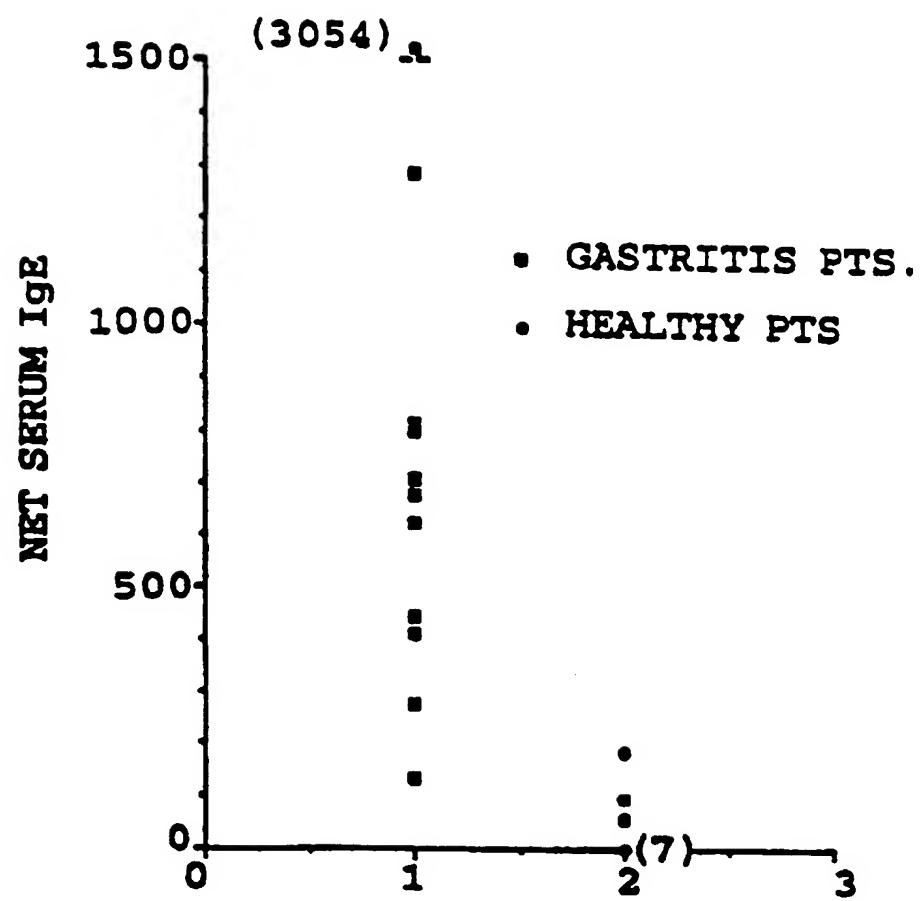
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FIGURE 3



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FIGURE 4



## INTERNATIONAL SEARCH REPORT

International Search Report No  
PCT/US 96/02625

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K39/00 A61K39/02 C07K14/00 C07K14/195 //A61K39/35,  
G01N33/53, C12N15/09

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO,A,95 17677 (CALENOFF EMANUEL J) 29 June 1995 see page 29, line 17 - page 31, line 37; claims 1-41 ---	1-6
X	WO,A,92 19970 (CALENOFF EMANUEL) 12 November 1992 see page 15, line 13 - page 18, line 5; claims 1-27 ---	1-6
A	ARCH OTOLARYNGOL HEAD NECK SURG, vol. 119, no. 8, 1993, pages 830-836, XP002008520 CALENOFF E. ET AL.: see the whole document ---	1-6 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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1

Date of the actual completion of the international search  
16 July 1996

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International	Application No
PCT/US 96/02625	

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ARCH MED RES, vol. 25, no. 2, 1994, pages 171-177, XP002008521 PEREZ O. ET AL: see abstract -----	1-6

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International cation No	PCT/US 96/02625
----------------------------	-----------------

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9517677	29-06-95	AU-B-	1512895	10-07-95
WO-A-9219970	12-11-92	AU-B-	667040	07-03-96
		AU-B-	2003692	21-12-92
		CA-A-	2109088	27-10-92
		EP-A-	0582672	16-02-94
		JP-T-	6507494	25-08-94